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Award Number: DAMD17-96-1-6015

TITLE: Actions and Substrates for the HER4 Tyrosine Kinase in Breast Cancer

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REPORT DATE: July 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Final (1 Jul 96 - 30 Jun 01)

4. TITLE AND SUBTITLE
Actions and Substrates for the HER4 Tyrosine Kinase in Breast Cancer

5. FUNDING NUMBERS
DAMD17-96-1-6015

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The EGF receptor family consists of four members (EGFR and HER 2, 3 and 4). One, HER2, is overexpressed in 20-25% of human breast cancers through gene amplification while another, the EGF receptor, is thought to be overexpressed or activated in poor prognosis breast cancers. Our objective was to determine whether the most recently discovered member, HER4, triggers a distinct, anti-proliferative and differentiation signal in breast cell lines and could therefore be a marker breast cancer with a better prognosis. After cloning the HER4 cDNA and constructing various chimeric and mutant receptors, we created novel breast cell lines expressing a variety of molecular constructs. These were used to conclusively demonstrate that HER4 activation stimulated anti-proliferative and differentiation responses in breast cell lines, even in the absence of HER2 signaling. Thus, HER4 is both necessary and sufficient to slow the growth of breast cancer cell lines. Studies in the last year allowed development of microarray technology to delineate the effect of HER4 signaling alone as compared to combined HER2, HER3, HER4 signaling in the control of gene expression. The experiments using the cell lines developed for this project have been performed, and hybridizations to 10,000 cDNA chip arrays have taken place. These arrays are awaiting bioinfomatic analysis. Lastly, we have investigated the role of EGFR vIII as a regulator breast cancer tumorigenesis. We have shown EGFR vIII is capable of phosphorylating HER 4 when co-expressed by transfection but does not appear to do so in the context of overexpression in breast cancer cell lines.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	100
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Abstract

The EGF receptor family consists of four members (EGFR and HER 2, 3 and 4). One, HER2, is overexpressed in 20-25% of human breast cancers through gene amplification while another, the EGF receptor, is thought to be overexpressed or activated in poor prognosis breast cancers. Our objective was to determine whether the most recently discovered member, HER4, triggers a distinct, anti-proliferative and differentiation signal in breast cell lines and could therefore be a marker breast cancer with a better prognosis. After cloning the HER4 cDNA and constructing various chimeric and mutant receptors, we created novel breast cell lines expressing a variety of molecular constructs. These were used to conclusively demonstrate that HER4 activation stimulated anti-proliferative and differentiation responses in breast cell lines, even in the absence of HER2 signaling. Thus, HER4 is both necessary and sufficient to slow the growth of breast cancer cell lines. Studies in the last year allowed development of microarray technology to delineate the effect of HER4 signaling alone as compared to combined HER2, HER3, HER4 signaling in the control of gene expression. The experiments using the cell lines developed for this project have been performed, and hybridizations to 10,000 cDNA chip arrays have taken place. These arrays are awaiting bioinfomatic analysis. Lastly, we have investigated the role of EGFR vIII as a regulator breast cancer tumorigenesis. We have shown EGFR vIII is capable of phosphorylating HER 4 when co-expressed by transfection but does not appear to do so in the context of overexpression in breast cancer cell lines.

Introduction

The human EGF receptor 2 gene (HER2) is amplified and overexpressed in 20-25% of invasive breast cancer (1). Moreover, many (but not all) observations indicate that poor prognosis breast cancers exhibit increases in EGF receptor content and/or an EGF receptor autocrine loop with the production of TGFa (2,3 and see Table 1). Both EGF receptor and HER2 can interact with each other, or with HER3 leading to growth and proliferation (4,5). Ligand (heregulin) dependent or independent activation of HER3 and HER2 signaling may be responsible for a proliferative signal. However, in certain cells, heregulin causes differentiation. It is possible that this heregulin-dependant differentiation is a product of HER2 signaling, but it may also involve signaling through the fourth member of the family HER4 (6,7). A third possibility is that in selected cells HER4 and HER2 hetero-dimerize and a combined HER2/HER4 signal causes heregulin-dependant slowing of growth and induction of differentiation. If HER4 signals stop proliferation and cause differentiation, then HER4 activation may slow the growth of breast cancer.

In addition, any HER4 anti-proliferative pathway would contain gene products with anti-growth functions that could be classified as tumor suppressors. Loss of such tumor suppressors could be a cause of breast cancer progression. Our tasks in this DOD grant were to obtain definitive evidence that HER4 provides a different biologic signal in breast epithelium, i.e., differentiation and/or anti-proliferation rather than proliferation, and to elucidate the pathway, or elements of the pathway, that differ between HER4 and the original three members of this receptor family (EGF receptor, HER2 and HER3). To this end, we created molecular reagents, and cell lines and devised technology which allowed us to prove that HER4 sends an anti-proliferative signal. These reagents and cell lines should provide us the wherewithal to isolate the unique members of the HER4 pathway as well as substrates phosphorylated by this tyrosine kinase in the coming years.

Body of Text

Using this DOD funding, we have firmly established that HER4, as a unique signaling agent, is capable of sending an anti-proliferative signal in breast epithelia cell including at least some breast cancer cell lines. In addition, HER4 is at least partly responsible for a differentiation signal including the synthesis of neutral lipids and the induction of E-cadherin, a transmembrane protein whose expression decreases in many aggressive breast cancers. To reach these conclusions, we have created multiple molecular constructs, several antibodies, and a number of unique, stably transfected cell lines. These will allow us to pursue subsequent objectives in the future, the elucidation of the signaling pathway by which HER4 sends its anti-proliferative and differentiation signals.

A. Creation of molecular reagents to study the growth promoting and differentiation effects of EGF receptor family members

Full-length HER4 cDNA clones, to our knowledge, were only available by materials transfer agreements with one of several companies. These type of agreements inhibit the free flow of information and we thought it important to make our own reagents. To this end, we cloned the cytoplasmic domain of the HER4 receptor and created a EGF receptor (extracellular and transmembrane domains) HER4 (cytoplasmic tyrosine kinase domain) chimera. We then decided for a number of reasons that we needed the entire HER4 molecule and therefore we isolated by PCR the HER4 extracellular domain again using RNA isolated from the MDA-MB 453 cells. After high fidelity PCR and exhaustive sequencing, we selected a clone that was wild type with respect to amino acid sequence and created a full length HER4 cDNA expressed using two vectors pcDNA and pLXSN. The latter can be used to make amphotrophic viruses capable

of infecting human breast cell lines, and in fact we used that strategy several times (see below). **Figure 1** schematically outlines clones and shows our strategy, which we have now accomplished, to create a kinase-dead full length HER4 and a HER4 kinase dead EGF receptor/HER4 chimera. These constructs were needed to achieve the aim of defining a HER4 signal. We showed that the chimera was capable of slowing growth both in the breast cancer cell lines and in 32D cells (see below). The appropriate control to show that this was due to the HER4 tyrosine kinase is a kinase-dead construct.

Another type of dominant negative construct was made, the extracellular domain anchored by a transmembrane domain of HER4. We've used the full length HER4 cDNA and placed a stop codon right after the tri-basic amino acid anchoring sequence in the juxtamembrane area of the molecule. High level expression could block the differentiation effect of heregulin in MDA-MB 453 cells and other cells in which heregulin causes differentiation. These experiments are planned.

Lastly in the area of creation of new reagents, we realize that since the HER4 signal is growth inhibitory it may be difficult to create cell lines expressing this molecule. Therefore we cloned the full length HER4, the chimera, as well as the kinase dead version of these two molecules and the HER4 dominant negative cytoplasmic domain into the tetracycline off system (**Fig 2**). This was felt to be one the best inducible promoter systems. However, we have not been successful in creating tet responsive gene expression in any of the breast cancer cell lines that we tried. The background expression remained high, the system was therefore not inducible. Fortunately, we were able to make new cell lines stably expressing HER4 (see below).

B. Action of the EGF receptor HER4 chimera and 32D cells

We created stably transfected neo-resistant 32D cell expressing the EGFR:HER4 chimera. Populations of chimeric transfected 32D cells underwent growth slowing in response to ligand (EGF). This was in line with our hypothesis that HER4 sends a differentiation and not a proliferative signal. We next selected two clones, which express different levels of the HER4 chimera. EHC-2 expresses a high level and EHC 11 expresses 10-20% as much HER4 chimera. **Figure 3** shows a FACS analysis of HER4 chimera expression. **Figure 4** shows the amount of tyrosine phosphorylated HER4 chimera in the two clones. The high expressor, EHC 2, exhibits constitutive autophosphorylation of HER4. The addition of EGF dramatically increases tyrosine phosphorylation but the most important aspect is that there is phosphorylation in the basal, non-ligand state. This correlates with the growth curve **Figure 5**, which demonstrates that EHC2 grows much more slower and that EGF blocks IL-3 dependent growth in this clone. The lower expressor, EHC clone 11, contains HER4 chimera and it can be activated in a EGF-dependent manner. However, there is no constitutive phosphorylation and this clone grows much faster (as shown in **Figure 5**).

C. Production of antisera

We have not been satisfied with the commercial antisera to HER4 (or HER3) and therefore made polyclonal antibodies by creating GST-fusion proteins and contracting with a commercial vender to produce antibodies. An original GST HER4 fusion protein immunization using the mid C-terminal region did not produce antisera with significant titer. We next re-immunize with a 100 amino acid C-terminal fragment of HER4 and have now obtained an excellent immunoprecipitating antibody. **Figure 6** shows that HER4 antibody will immunoprecipitate tyrosine phosphorylated HER4 from clone 2. The antisera is sufficient to detect phosphorylated HER4 in ligand treated breast cell lines that express modest HER4 levels. We have recently obtained anti-HER3 antibodies with excellent immunoblotting properties using a GST HER3 C-terminal fusion protein as immunogen.

In addition, we have placed the extracellular domain of HER4 into the p-FASBAC vector and have created a HER4 extracellular domain baculovirus (**Fig 7**). This soluble protein has the 6 histidine tag at the end and therefore we can purify the extracellular domain using nickel columns. This immunogen was used by a contractor in an attempt at monoclonal antibody production. Our aim was to create a monoclonal antibody capable of recognizing the human HER4 in paraffin-embedded sections. In this manner, we would be able to do translational research with archived tissues allowing us to determine whether the HER4 expression level is an independent prognostic variable for breast cancer survival or whether it is a modifier of HER2 predicted prognosis. Unfortunately, our contractor was not successful in making monoclonal antibodies. We have obtained as a gift of Mark Slikowski of Genentech, several candidate HER4 monoclonals that we will test for the desired properties.

D. Studies of the SUM 44 cell line

We work with a number of breast cancer cell lines, including those initiated in Steve Ethier's laboratory at the University of Michigan. **Figure 8** shows that treatment of various cell lines with heregulin resulted in growth suppression in several lines including the SUM 44 cell line. The MDA-453 cell line that has been shown by others to exhibit heregulin-dependent differentiation does not do so in our hands. Investigation showed that our clone has a high level of basal (non-ligand-dependent) HER4 tyrosine phosphorylation. The cell line grows slowly due to a heregulin autocrine loop. Our work analyzing HER4mRNA (see later section) shows that only cells that express HER4 mRNA undergo heregulin-dependent growth slowing. **Figure 9** shows the SUM44 anti-proliferative response to both heregulin and HB-EGF. Both ligands (from two separate ligand families) are capable of activating HER4 tyrosine phosphorylation. **Figure 10** shows the extent of HER4 tyrosine phosphorylation in SUM 44 and MDA 453 cells treated with heregulin or HB-EGF. It is clear that heregulin stimulates a HER4 signal as a judge by tyrosine phosphorylation to a greater extent than HB-EGF. These experiments were accomplished using our polyclonal HER4 antisera. This antiserum is excellent for immunoprecipitation and western blotting and, we believe, is superior to any commercial antibody on the market. **Figure 11** shows that SUM44 cells treated with heregulin differentiate as judged by morphology and their elaboration of neutral lipids indicating that the HER4 tyrosine phosphorylation results in a true differentiation phenotype. **Figure 12** shows a fluorescence-activated cell sorter analysis of SUM44 cell neutral lipid content that increases with heregulin treatment. Other assays (not shown) demonstrate that DNA content increases in cells treated with heregulin. Cells enlarge as they differentiate, as well as double their DNA content. This could be interpreted as cell cycle arrest at the G2M border, but it could also be a result of polypliod development (endoreduplication) in differentiated breast cell line. Lastly, stable transfection of the kinase dead HER4 cDNA into SUM44 cells blocked heregulin-dependent growth slowing (**Fig 13**). This shows that HER4 is necessary for the ligand-dependent anti-proliferative response.

E. Development of a matched pair of cells expressing or not expressing HER4

To give additional proof that the HER4 signal is anti-proliferative, we created matched pair of transfected cells, which expressed or did not express HER4. We screened available cell lines and determined that the SUM102 cell line did not express the HER4 receptor. Our HER4 molecular constructs (and vector alone conferring neo resistance) were packaged as an amphotrophic retrovirus and used to infect several hard to transfect cell lines such as the SUM 102 cell. G418-selection resulted in the creation of two types of cells, SUM 102-HER4 and SUM 102 neo vector. **Figure 14** provides convincing evidence in SUM 102 neo vector cells did not exhibit HER4 activation when treated with heregulin. On the other hand, clones were

selected in which heregulin dramatically stimulated HER4 tyrosine autophosphorylation. Thus, we have created a matched pair of cell lines expressing HER4 or vector alone.

These cells were tested for their growth inhibitory response. **Figure 15** demonstrates an anti-proliferative response of HER4 containing cells to heregulin. The cells (SUM102 HER4) also show an anti-proliferative response to HB-EGF (not shown). **Figure 16** shows the results of the neutral lipid FACS assay we have developed. The cells treated with or without heregulin were stained with Nile Red and then analyzed by FACS. Heregulin produced a significant shift in Nile Red positive cells providing evidence that not only did heregulin slow the growth of the cells expressing HER4, but heregulin resulted in differentiation. As expected, control SUM 102 neo vector cells did not respond to heregulin with differentiation. Another assay for differentiation is the induction of E-cadherin. This gene is not expressed in SUM44, so we could not assess the ability of a HER4 ligand to induce E-cadherin in those cells. However, in transfected SUM102 HER4 cells, heregulin increased E-cadherin expression; this did not occur in neo vector SUM102 cells (**Fig 17**).

F. Demonstration that HER4 sends an anti-proliferative signal in the absence of HER2

It has been known for over 10 years that when certain cells are treated with heregulin, they differentiate; in fact, one of the original groups that isolated a putative ligand for HER2 called the protein, the neu differentiation factor or NDF because the cell systems that they studied differentiated when treated with the factor during purification (8). Virtually all of the studies investigating heregulin-dependent cell growth or differentiation using breast or ovarian cell lines suffer from the same problems. Cells that differentiate express both HER4 and HER2, making it difficult, if not impossible, to distinguish whether HER4 by itself slows growth or causes differentiation.

To address this, we have adopted a technology pioneered by Nancy Hynes (9). She has taken a single chain antibody to HER2 fused to an endoplasmic reticulum (ER) targeting sequence. The expression of this intracellular, single chain antibody results in trapping HER2 intra-cellularly before it can be transported to the plasma membrane where it could signal in a heregulin-dependent manner. We obtained the sc anti HER2 antibody from Dr. Hynes in a vector, which encodes resistance to the antibiotic puromycin. We created two matched pairs of cell lines. The first was the SUM 44 cell, which expresses both HER4 and HER2. Transfection of cDNAs encoding either vector or vector plus containing scHER2 antibody into SUM 44 followed by selection created two populations of puromycin resistant cell lines. **Figure 18** shows that the addition of heregulin to the vector alone expressing cells results as expected in heregulin-dependent HER2 phosphorylation, as shown by HER2 immunoprecipatation followed by p-tyr immunoblotting. However, the addition of heregulin to the cell line expressing the single chain antibody can not stimulate HER2 tyrosine phosphorylation because HER2 was not at the cell surface (**Fig 18**). We have done immunofluorescence and have shown that the reason why HER2 was not activated was because all the HER2 was found intracellularly, not on the cell's membrane (data not shown). Thus, we have successfully abolished HER2 signaling in these cells.

None the less, addition of heregulin to cells without a HER2 signal produces an anti-proliferative effect as does the addition of HB EGF in SUM44 (**Fig 19A**). As in all other situations, HB EGF is less effective than heregulin in creating anti-proliferative response, because it does not stimulate HER4 as well as heregulin. This shows that heregulin-dependent anti-proliferations does not involve HER2 and that the HER4 signal alone is sufficient. The latter follows because HB-EGF, which would not trigger HER3 tyrosine phosphorylation, also slows cell growth.

An even more complex set of cell lines were created by infecting the neo-resistant SUM102 HER4 cells (containing the neo-resistant vector or the vector encoding both neo and HER4) with a second cDNA encoding either puromycin-resistance alone, or puromycin-resistance plus the single chain HER2 antibody. This was accomplished by packaging retroviruses with the puromycin resistance vectors and infecting cells. The cells were then selected using both selectable markers (G418 and puromycin). Once the cell lines were created, we were able to show that heregulin-dependent, anti-proliferative response was retained when HER2 signaling was abolished (**Fig 19B** SUM102-HER4-5R). The results are conclusive; a heregulin-dependent, anti-proliferative effect is fully operable in the absence of any HER2 signaling.

The production of neutral lipids in SUM44 and SUM102 cells and the induction of E-cadherin in SUM102 cells, all markers of differentiation, are less well induced in the cell lines without cell surface HER2 signaling (the SUM44 and SUM102 5R bearing cells). However, this is in part because the basal level of lipids goes up with the withdrawal of cell surface HER2 i.e HER2 may contribute to the undifferential state. Thus we can say reasonably that HER4 is necessary and sufficient for an anti-proliferative signal, and that HER4 is at least necessary for the differentiation signal. However, HER4 might not be totally sufficient for all aspects of differentiation.

Results showing the HER4 is necessary are confirmed in SUM44 cells in which we have transfected dominant negative kinase dead HER4 constructs (see **Fig 13**). This abrogates the HER4 signal and prevents the anti-proliferative response. This is an area in which we will continue to work. We will try to tease apart a potential role of hetero-dimerization between HER4 and HER2 signals in the differentiation response as opposed to the HER4 alone sending an anti-proliferative signal. This next year we will use our extension to perform microarray analysis of gene expression using cells with a "pure" HER4 signal versus a mixed HER4 HER2 signal.

As a part of these studies, we have demonstrated that heregulin-dependent, HER4 activation in the absence of HER2 is perfectly capable of activating MAP Kinase and AKT. These experiments are the beginning of our analysis of pathways directly stimulated by HER4.

Additional studies are beginning to get to the issue of mechanism that is by definition complex. First, using the kinase dead HER4 SUM44 cells, we've showed in Fig 4 of the accompanying *Molecular and Cellular Biology* manuscript that expression of the kinase dead HER4 construct abrogated the heregulin-dependent anti-proliferative response in these cells. When we looked at the tyrosine phosphorylation of HER4, we showed that tyrosine phosphorylation of HER4 actually increased by four to five fold in the kinase dead HER4 cells as the result of the vast overexpression of the kinase dead HER4 construct. The increase in tyrosine phosphorylation of the kinase dead construct must be due to heregulin- and HER2-dependent - HER4 phosphorylation, and yet the biologic activity is abrogated. Taken in context of the other results, this shows that the sites on HER4 phosphorylated by HER2 (which are dramatically elevated in the SUM44 kinase dead expressing cells) must not be the ones that are responsible for sending the signal to block cell growth. This cell line will give us other options for performing cDNA microarray analysis to try to determine which signals emanating from HER4 (either kinase active or kinase dead) are responsible for antiproliferative or other HER4 effects.

Additional experiments with the 5R cells have indicated that HER3 phosphorylation, at least in this cellular context, is predominately a result of HER2 HER3 complexes and not HER4 HER3 complexes. If one examines Figure 7 of the accompanying *Molecular and Cellular Biology* manuscript, one sees that the expression of the single chain antibody not only eliminates HER2 signaling from the SUM44 cells but also virtually eliminates HER3 – and dramatically decreases HER4 signaling. This shows that HER3 and HER2 are not involved in the antiproliferative response. At this juncture we cannot fully pull apart the antiproliferative and

the differentiation response. The pBABE and 5R cells are transfected with vectors that involve resistance to puromycin. These cells grown in puromycin, even without heregulin, exhibit some expression of Nile red oil droplets i.e., the response to puromycin (presumably its toxic effect) results in some level of breast cell differentiation. Therefore, we can say that HER4 signal alone is responsible for the antiproliferative response, but we are not prepared at this time to say that HER2 and/or HER3 do not contribute to the differentiation response.

G. Quantitative PCR for HER4 mRNA levels

A long- term objective of this project is to understand the potential of HER4 as a marker of breast cancer prognosis and perhaps even response to therapy. Multiple translational studies have attempted to measure the amount of HER1 (EGFR) and particularly HER2 expression in breast cancer. It is widely accepted that the HER2 gene amplification occurring in ~20% of women with breast cancer is both a poor prognostic sign and a finding that has therapeutic import. The roles of HER1, HER3, and HER4 as individual entities are much less well defined and activated receptor signaling as a predictive function is even harder to approach.

There are multiple articles describing the expression of EGF receptor and its relationship to prognosis in breast cancer (see Table 1). These data in the aggregate are confusing and certainly not definitive. Studies of HER3 and HER4 expression are fewer and in general, have not been done on large data sets (10-12). Several of the small clinical studies of HER4 expression favor the idea that HER4 is a good prognostic sign;(12) this would support the central hypothesis of this proposal that HER4 is a differentiation signal. This finding is compatible with data from our group and others. Since EGF receptor family members heterodimerize and the outcome of the signals may depend upon the partners heterodimeric complex (eg. HER2/HER3 vs. HER2/HER4), it is important to analyze the expression of all 4 family members in breast cancers to fully understand the family's biology and prognostic import. This is a daunting task. The best method would be to have antibodies to all four members that are useful in archival, paraffin-embedded samples. Good antibodies for all members are not available. Thus, while there are issues regarding the interpretation of the relationship between mRNA levels and protein levels, we have chosen to use new quantitative PCR methods for accurately quantitating family member mRNA levels (ABI 7700, "Taq man").

Our experience shows that the quantitative PCR assay is extraordinarily accurate and reproducible once one has extracted and accurately measured RNA. During the last year, we have created PCR primers and specific "Taq man" probes for the EGF receptor, HER2, HER3, and HER4. We have cloned the 4 human family member cDNAs into vectors that allow us to transcribe RNA from the vector to make more mRNA populations that can be used to develop standard curves for the Taq man assay (**Fig 20**). We have developed accurate, highly reproducible, standard curves for each of the four molecular species. We have miniaturized the assays so that we can use very small (ng) amounts of total RNA (without the need for poly A selection) to measure mRNA content of HER1-4. **Figure 20** shows representative standard curves. **Figure 21** demonstrates the HER4 mRNA expression in a panel of breast cell lines that we have examined. One of those lines, SUM44, was used for multiple experiments.

SUM44 expressed reasonable HER4 levels without exhibiting non-ligand-dependent autophosphorylation. **Figure 22** shows the levels of HER1-4 in a panel of cell lines. It is interesting to note that all cells express HER3. HER3 levels are always greater than HER4 levels in this panel of cell lines. The y axis in Figures 21 and 22 differs by ten-fold so that we could show the high copy levels of HER2 in some of the cell lines.

With other funding, we have used quantitative PCR to assess EGFR family member including EGFR VIII mRNA levels in human breast cancers. We have extracted mRNA from

over ~30 matched pairs of operative samples in which breast cancers and matched "normal" tissue adjacent to the tumor was obtained. We are also working on extracting RNA from breast cancer core biopsies, so that we can follow EGF receptor family mRNA expression before and after chemotherapy in our neo-adjuvant protocols. The results of HER4 and EGFR VIIII analysis in breast and prostate cancer are included in this report for completeness as an Appendix after the *Molecular and Cellular Biology* paper. This particular DoD grant will end before these human core biopsy human studies are begun, but the current grant has funded the development of a technique for HER4 (and other EGFR family member) mRNA assessment.

H. cDNA Microarray of HER4 signaling

In collaboration we have been developing cDNA microarray capabilities at the UNC Lineberger Comprehensive Cancer Center this year. We have purchased equipment, and Research Genetics cDNA clones that will allow us eventually to print a 20,000 cDNA microarray slide. Currently, we are printing 10,000 cDNA microarray slides. These technical capabilities are now in place, and we have done the first major experiment, which is to analyze SUM44 cells transfected either with vector (pBABE) or 5R (a single chain HER2 antibody). Treating these two sets of cells with heregulin gives us either a combined HER2, HER3, HER 4 signal (vector alone) or a pure HER4 signal (the 5R cells). One can examine the signaling in these two by looking at Figure 7 of the *Molecular and Cellular Biology* paper. Large-scale cell culture has been accomplished, and RNA has been made and poly A selected. Hybridizations from all samples have been performed on the 10,000 cDNA array after labeling 2 RNAs with different fluorescent probes. The hybridization has been viewed and is of high quality with clear-cut changes in gene expression changes, both positive and negative, between the two cells lines seen by eye. Scanning and bioinformatics analyses are now in progress. These data should give us insight into the signaling systems that are stimulated by HER4 alone versus those that are stimulated by the combination of HER2, HER3, HER4 signaling.

I. Investigation of EGFR vIII

Several investigators have indicated using an antibody that a truncated rearranged form of the EGF receptor (referred to as EGFR vIII), is expressed in over 50 percent of human breast cancers. This product is clearly expressed in glioma cells where the EGF receptor gene is amplified and rearranged. This gene re-arrangement eliminates exons 2-7 in the extracellular domain and results in an EGF receptor that no longer binds EGF but is constitutively active. This would be an extremely important finding in human breast cancer if it were substantiated by other technologies. Therefore, we began to investigate the biochemical actions of EGFR vIII in breast cells. With respect to the DoD grant, we particularly wanted to determine whether this constitutively active EGF vIII receptor could phosphorylate HER4 and if it did so, what would the biological consequences be. The experiments that we performed are enclosed in a manuscript that has been submitted for publication. The first thing that we did was to co-express EGFR vIII with kinase inactive EGF receptor, kinase inactive HER2, the naturally kinase inactive HER3, and kinase inactive HER4. Each of these EGF receptor family cDNA clones was made kinase inactive by site-directed mutagenesis in our laboratory. Co-expression showed definitively that the overexpressed EGFR vIII had the capability of tyrosine phosphorylating kinase inactive HER1, 2, 3, and 4. To look at this in the biologic context, we used retroviral infection to create two cell lines, expressing EGFR vIII and the appropriate vector controls. The first line was SUM44, which does not express the EGF receptor, but does express HER 2, 3, and 4. The second line was the normal MCF10A cell that is immortalized but not transformed. In the SUM44 line over-expressing EGFR vIII and the mutant EGFR did not phosphorylated HER4, and there was no effect on the growth curves, either positively or negatively. These transformed cells grow in soft agar. EGFR vIII expression in MCF10A cells also did not result in the tyrosine phosphorylation of HER4 but did result in transformation of these cells. Thus,

while it is true that EGFR vIII can effect normal breast epithelial cells and even transform them in culture, we do not believe that this is expressed in human breast cancers and, therefore, is not a target for therapeutic advances.

Key Research Accomplishments

- Cloning of full length HER4 cDNA
- Cloning of a chimeric EGF receptor (extracellular and transmembrane domains) HER4 (cyloplasmic domain cDNA)
- Site directed mutagenesis of above cDNAs to abolish tyrosine kinase activity creating kinase dead, dominant negative constructs
- Creation of retroviral vectors expressing HER4 and dominant negative HER4 constructs
- Creation of polyclonal antisera against the C terminis of human HER4 and HER3
- Creation of a his-tagged baculovirus expressing HER4 extracellular domain
- Creation of 32D myeloid cell lines expressing the EGF receptor HER4 chimera
- Demonstration that the EGFR HER4 chimera 32D line slows growth in response to ligand
- Demonstration of heregulin-dependent HER4 tyrosine phosphorylation in some breast cancer cell lines
- Demonstration that heregulin-dependent breast cell line differentiation only occurs in HER4 expressing cells
- Creation of matched pair of cell lines expressing vector or full length HER4 from a cell line, SUM102, that did not express HER4
- Demonstration that HER4 signaling in a cell line made to express HER4 causes differentiation
- Creation of a breast cell lines expressing an EGFR:HER4 chimera and demonstrating EGF-dependent differentiation
- Creation of cell lines with dominant negative (kinase dead) HER4 and demonstrating that this dominant negative construct blocks heregulin-dependent differentiation in a HER4 expressing line
- Demonstrating that kinase dead HER4 can be tyrosine phosphorylated in a HER2-dependent manner but does this not send an anti-proliferative signal.
- Creation of a new flow cytometry assay to assess neutral lipids in differentiating breast cell lines
- Demonstrating that heregulin-dependent HER4 activation upregulates E-cadherin, an additional assay of HER4-dependent differentiation
- Creation of pairs of cell lines expressing either vector (p BABE) or a cDNA expressing a singe chain (sc)HER2 antibody with an endoplasmic reticulum (ER) targeting sequence
- Demonstrating that expression of scHER2 antibody with an ER targeting sequence prevents cell surface expression of HER2 and heregulin-dependent HER2 activation

- Demonstrating that in the absence of HER2 signaling heregulin still has an antiproliferative effect in cells with HER4 signaling. This demonstration meets one of the two major objectives of this grant
- Demonstration of heregulin-dependent MAPK and AKT activation in HER4 expressing cells even in the absence of HER2 signaling
- Demonstrating that heregulin-dependent HER3 phosphorylation is dependent upon HER2 and not HER4.
- Creation of an ultra sensitive quantitative PCR assay for HER4 mRNA levels and a similar assay for EGFR, HER2 and HER3 RNA levels
- Use of the quantitative PCR to measure the level of HER1-4 mRNA in a panel of breast cancer cell lines
- Demonstrating that only cells expressing HER4 and not expressing autocrine heregulin pathways exhibit a heregulin-dependent antiproliferative effect
- Initiation of studies of quantitative PCR in samples derived by laser capture microdissection.
- Setting up cDNA microarray analysis of cells and studying mRNA expression after heregulin-dependent HER2, HER3, and HER4 tyrosine phosphorylation as compared to isogenic cells exhibiting only HER4 tyrosine phosphorylation.
- Isolation and poly A selecting mRNA from SUM44 vector alone or 5R cells and hybridizing the generated cDNA to 10,000 cDNA microarrays.

Outcomes

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Conclusions

We have demonstrated that HER4 signaling is both necessary and sufficient to deliver a ligand-dependent, anti-proliferative signal. These conclusions have been reached with members of two different ligand families and at least two human breast cancer cell lines. The majority of breast cancer lines do not express HER4 and our hypothesis is that its anti-proliferative effect is selected against when breast cancer cells are forced to grow out as established lines in culture. In fact, HER4 may be lost in breast cancer progression for the same reason. We have made

multiple molecular construct for those studies and used these to create new cell lines that can be used to study a "pure" HER4 signal in the absence of HER2, or a combine HER4, HER2 signal. We will use the models created during the next year's extension, to perform more in depth studies of HER4 signaling in the presence and absence of HER2. We have initiated microarray "gene chip" analysis on these cell lines to detect the families of genes expressed in response to the anti-proliferative HER4 signal. This approach will begin a secondary objective which, is to determine whether there are any tumor suppressor genes in HER4 anti-proliferative signaling pathway that can be used along with HER4 expression as prognostic variables in predicting the outcome of breast cancer and its therapy. Analysis of the RNA isolated from isogenic cells with HER2, HER3, and HER4 signaling versus those only signaling via HER4 will help launch a new set of investigations in HER4 signaling.

In addition, we have set up a quantitative PCR assay for HER4 so that we can accurately assess the level of mRNA expression for HER4 (as well as the EGF receptor, HER2 and HER3). Multiple laboratories have shown combinatorial interaction between these receptors and their ligand families. A full understanding of breast cancer prognosis and biology will only be obtained when we know the expression and, in fact, the level of activation of the multiple family members. Our quantitative PCR assay is being miniaturized, and we hope to be able to use laser capture microdissected material during the next year to assess accurately EGFR and HER2, 3 and 4 expression in breast cancer samples versus adjacent normal breast epithelium.

In summary, we have met one major objective by determining that HER4 is indeed an anti-proliferative and differentiation signaling receptor. We have created the molecular and cellular reagents to allow us to dissect a pathway of anti-proliferation and potentially detect tumor suppressor genes. Lastly, we have created a quantitative PCR assay using breast cell lines. In the future, this assay will allow us to screen breast cancers in clinical translational studies to determine whether our overall hypothesis regarding the biology of the HER4 receptor is reflected in the prognosis of breast cancers expressing HER4.

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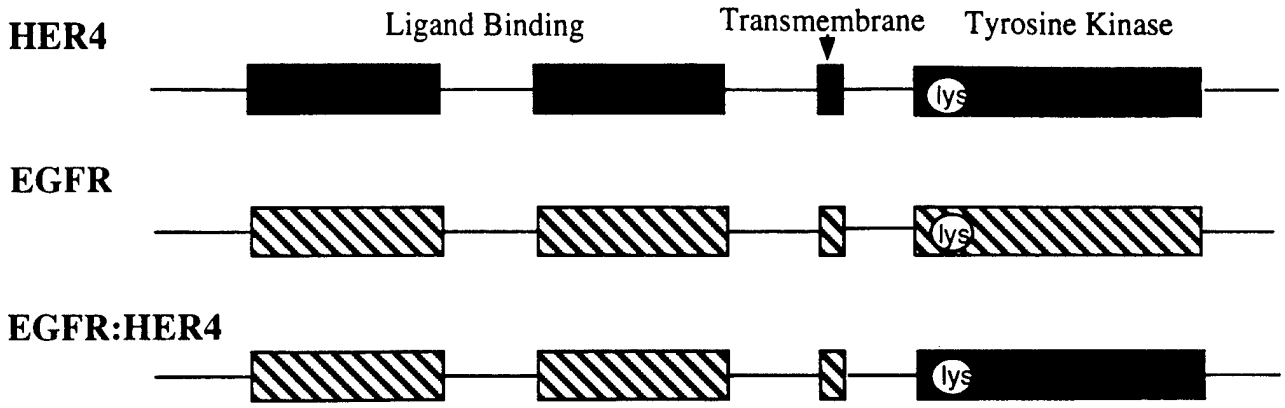
Table 1

Epidermal Growth Factor Receptor (HER1) Levels in Breast Carcinomas and Related Outcomes Analysis

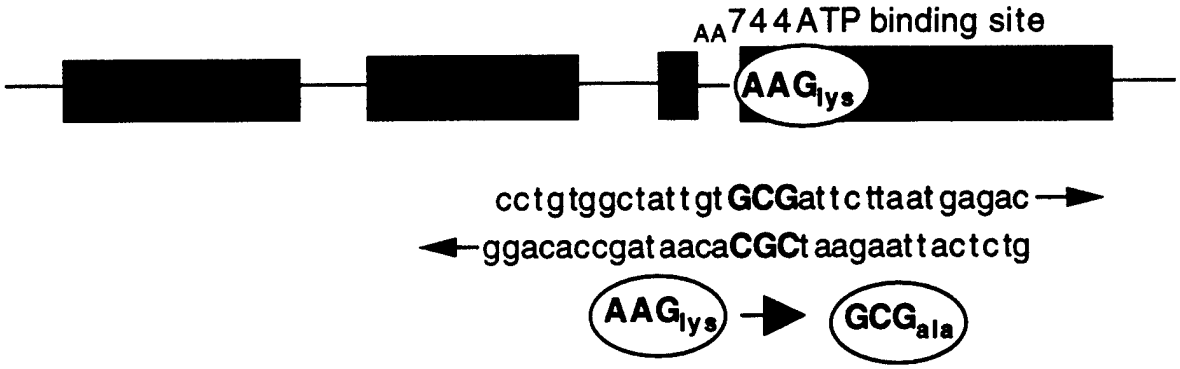
Author	Year	N	Method	Cut-Off	% Pos.	Univ. RFS	Univ. OS	Multi Var. RFS	Multi.Var OS
Rios[13]	1988	179	LB	1 fmol/mg	43	<0.05			
Costa[14]	1988	376	LB	10 fmol/mg	---				
Grimaux[15]	1989	55	LB	5 fmol/mg	33	NS	0.05	0.01	<0.01
Foekens[16]	1989	203	LB	None	91(any +)	NS		NS	
Spyratos[17]	1990	109	LB	10 fmol/mg	34	0.05		0.03	
Lewis[18]	1990	90	IH	>2+	14	<0.003		0.04	
Toi[19]	1991	135	LB	1 fmol/mg	41	<0.05			
Hawkins[20]	1991	120	LB	1 fmol/mg	43	NS	NS	NS	NS
Nicholson	1991	231	LB	10 fmol/mg	35	<0.001	<0.001	0.03	NS
Osaki[22]	1992	115	LB	1 fmol/mg	35	<0.01			
Bolla[22]	1992	232	LB	3 fmol/mg	51	NS		NS	
Shrestha[23]	1992	50	IH	Any	44	<0.05	<0.05		
Gasparini[24]	1992	164	IH	>5% cells	56	0.003	NS	0.0049	
Fox[25]	1993	370	LB	20 fmol/mg	47	0.03	0.05	0.03	NS
Koenders[26]	1993	376	LB	50 fmol/mg	22	0.03	0.002	NS	NS
Murray[27]	1993	107	mRNA	+ or ++	51	NS	NS		
Hawkins[28]	1996	215				NS	NS	NS	NS

LB = ligand binding. **IH** = immunohistochemistry. **Univ**= univariate. **RFS** = Recurrence Free Survival. **OS** = Overal Survival.



HER4 and EGFR:HER4 kinase dead

HER4 kinase dead



EGFR:HER4 kinase dead

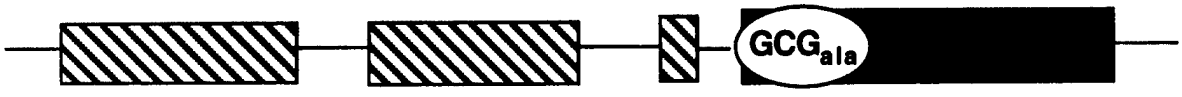


Figure 1. Expression vectors for use in HER4 experiments. We isolated the HER4 extracellular domain and constructed a full length HER4 cDNA. In addition, we created two kinase dead mutants by site-directed mutagenesis.

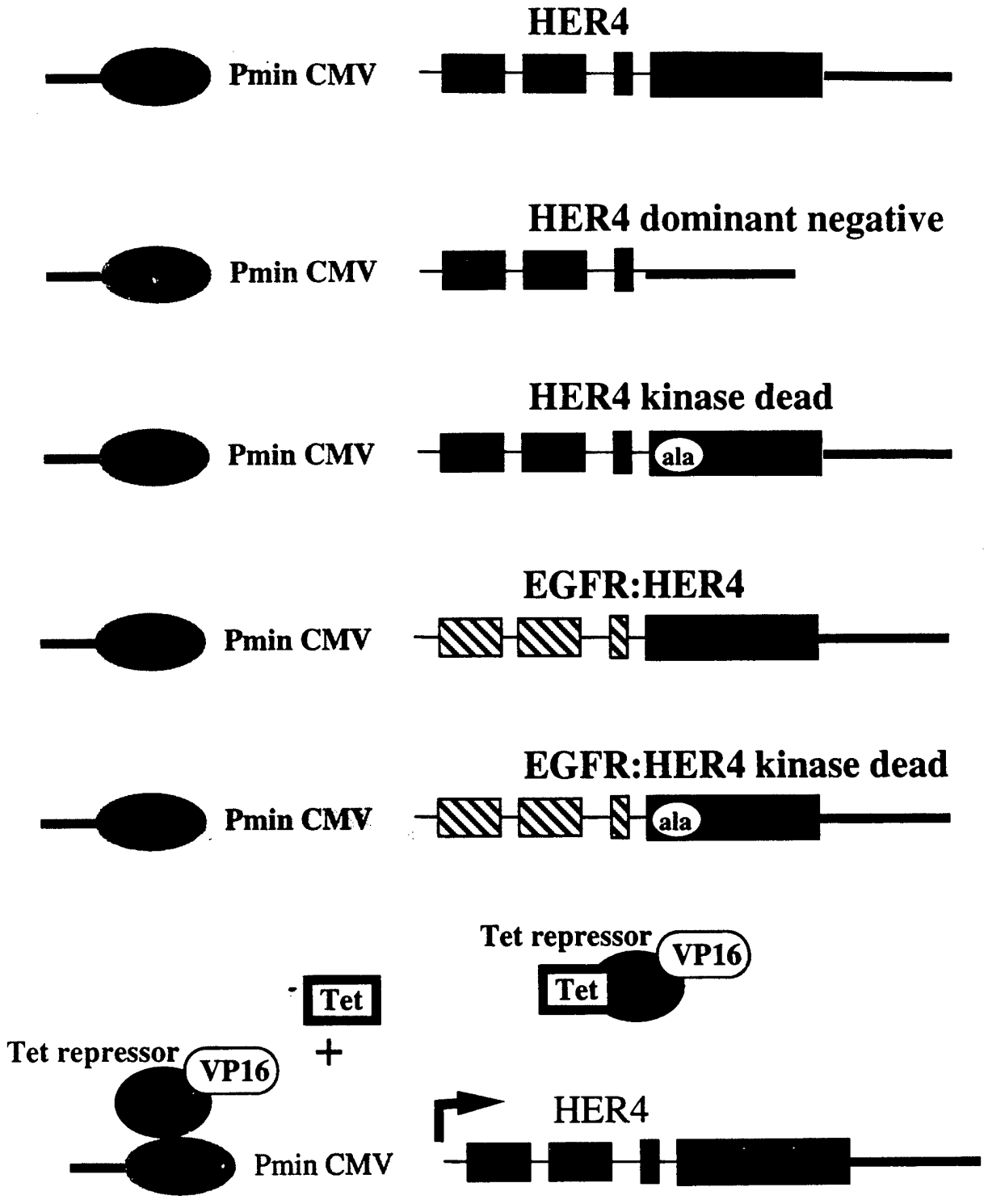


Figure 2. Inducible promoter for HER4 constructs. Each of the important HER4 cDNA's placed in the "Tet off" vector which would allow the creation of stable transfected lines with tightly-controlled HER4 construct expression.

vector control

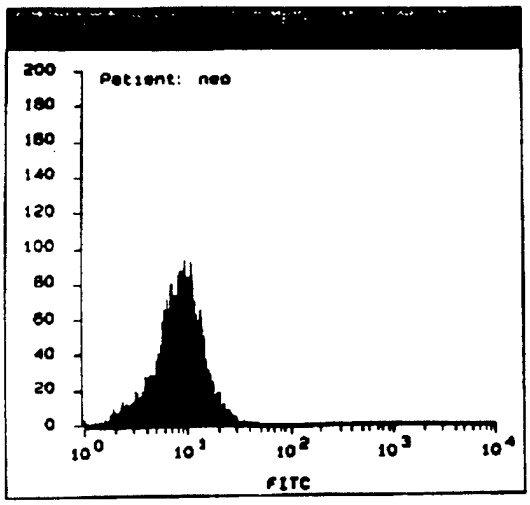
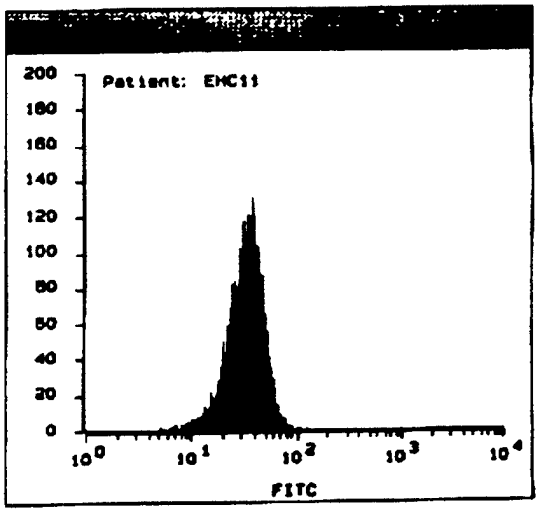
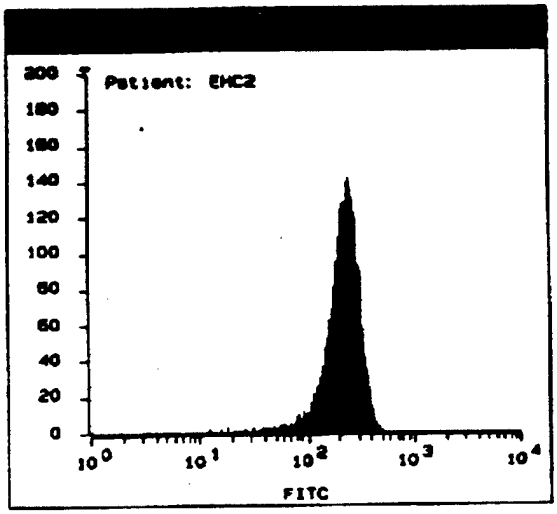


Figure 3. Fac Scan analysis shows that we have created two 32D cell clones with constitutively high (EHC2) and low (EHC11) levels of expression. 32D cells were washed and incubated with anti-EGFR antibody, followed by an incubation with anti-rabbit FITC conjugated secondary antibody. The cells were washed once more and analysed by flow cytometry for FITC staining.

EGFR:HER4 clone 11



EGFR:HER4 clone 2



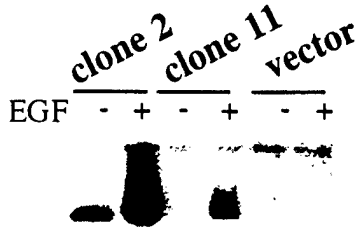


Figure 4. The high (EHC2) and low (EHC11) expressing clones exhibit different levels of ligand-independent (-EGF) and ligand-dependent(+EGF) tyrosine phosphorylation. 32D cells were removed from IL3 containing medium for 3 hours. The cells were then stimulated with 0.1 µg/ml EGF for 90 seconds, washed, lysed and immunoprecipitated with anti-EGFR antibody. The immunoprecipitate was then run on a 8% SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. Clone 2 expresses the EGFR:HER4 chimera at 5-10 times the level of clone 11. The chimera is constitutively activated but can be further stimulated by EGF. Whereas clone 11 expresses the chimera to a lower level and is active only with the addition of EGF.

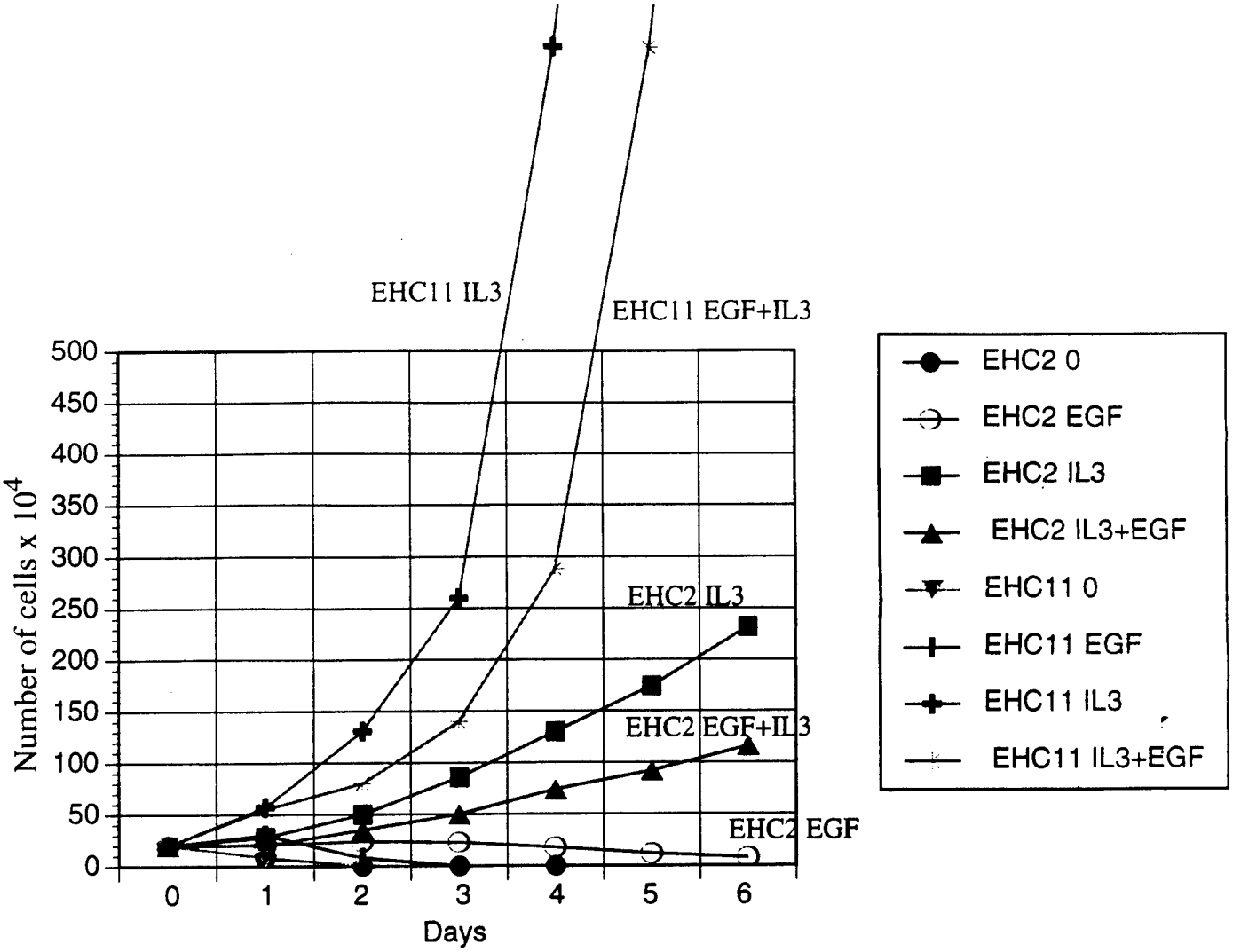


Figure 5. Growth curves of EHC expressing 32D clones. High expressing EHC2 clone shows a different growth pattern compared to low expressing EHC11 clone. EHC11 cells grow exponentially with the addition of IL3, and slightly slower with both IL3 and EGF. With no addition or addition of EGF alone the EHC11 cells die quickly; all are dead within two days. Thus the level of HER4 chimera expression in EHC11 is insufficient for biological signalling. In contrast, EHC2 which expresses EHC at high levels and exhibits an autoactivated HER4 kinase, grows slowly in the presence of IL3, and even slower with the addition of both IL3 and EGF. However, addition of EGF prevents cell death, and EHC2 cells remain viable many days after EHC11 cells have died.

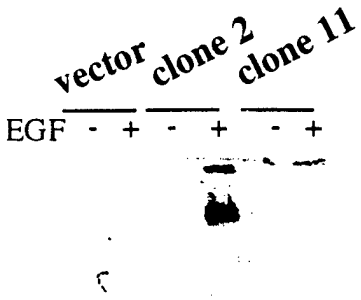


Figure 6. A GST-HER4 fusion protein was used to raise anti-HER4 antibody. 32D cells were removed from IL3 containing medium for 3 hours. The cells were then stimulated with 0.1 μg/ml EGF for 90 seconds, washed, lysed and immunoprecipitated with anti-HER4 antibody. The immunoprecipitate was then run on a 8% SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. In addition, when the blot is overexposed a faint band is seen with clone 11. Thus we have a specific anti-HER4 antibody.

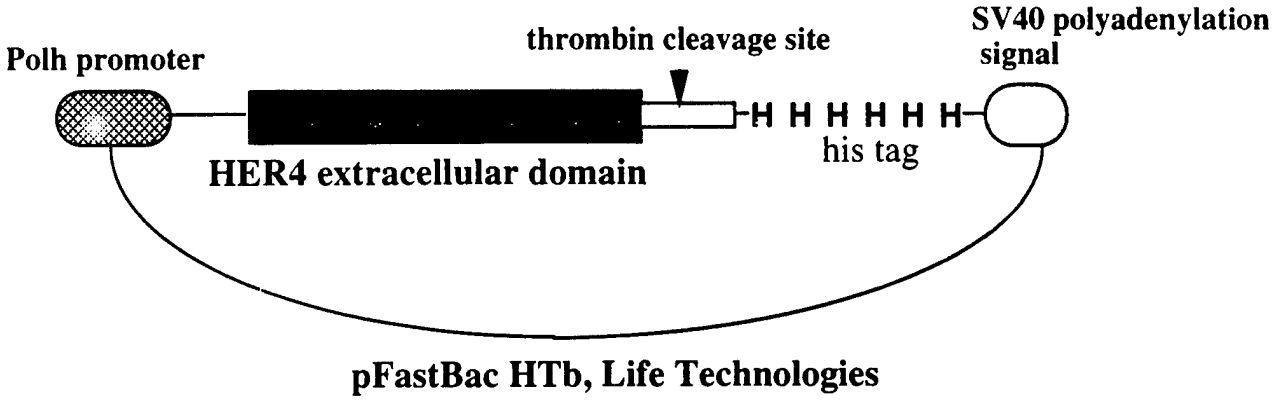


Figure 7. Creation of an extracellular domain HER4 baculovirus. HER4 extracellular domain was cloned into pFastBac baculovirus expression plasmid. Purification with Ni^{++} columns that bind to the 6-His tag will provide immunogen for monoclonal antibody formation.

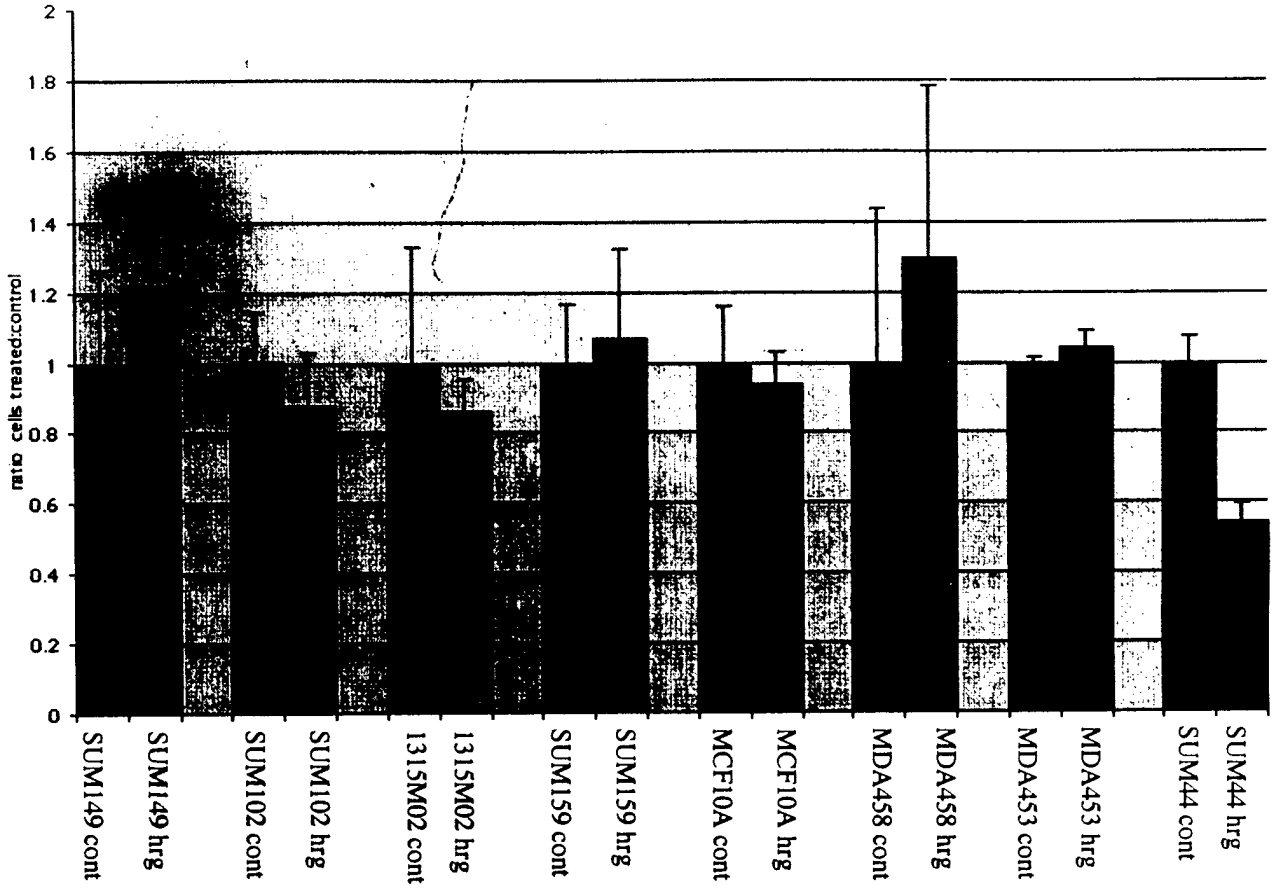


Figure 8. Proliferative response of human breast cancer cell lines to heregulin. Cells were plated at a density of $5x10^5$ cells per well in 6-well plates and grown in the presence or absence of 10 ng/ml heregulin B1 for 3 media changes (7 days), and the number of cells counted. The ratio of number of cells grown in the presence vs. in the absence of heregulin is shown. Error bars represent standard deviation of at least 3 experiments. SUM44 cells demonstrated the most pronounced effect of heregulin, an antiproliferative effect.

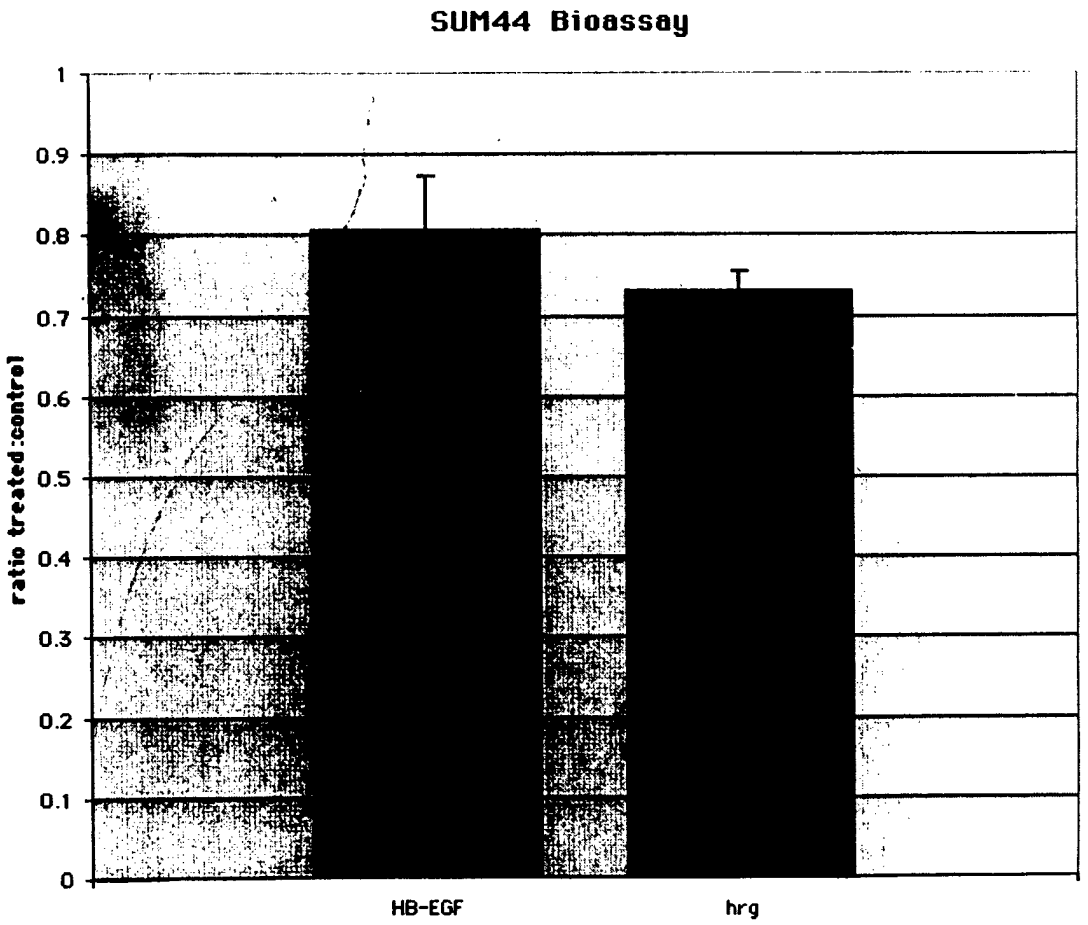


Figure 9. Anti-proliferative response to HB-EGF. SUM44 cells were plated at a density of $5x10^5$ cells per well in 6-well plates and grown in the presence or absence of 10 ng/ml heregulin B1 or 100 ng/ml HB-EGF for 3 media changes (7 days), and the number of cells counted. The ratio of number of cells grown in the presence vs. in the absence of ligand is shown. Error bars represent standard deviation of at least 3 experiments. Like heregulin, HB-EGF caused an anti-proliferative effect, although not to as great a degree.

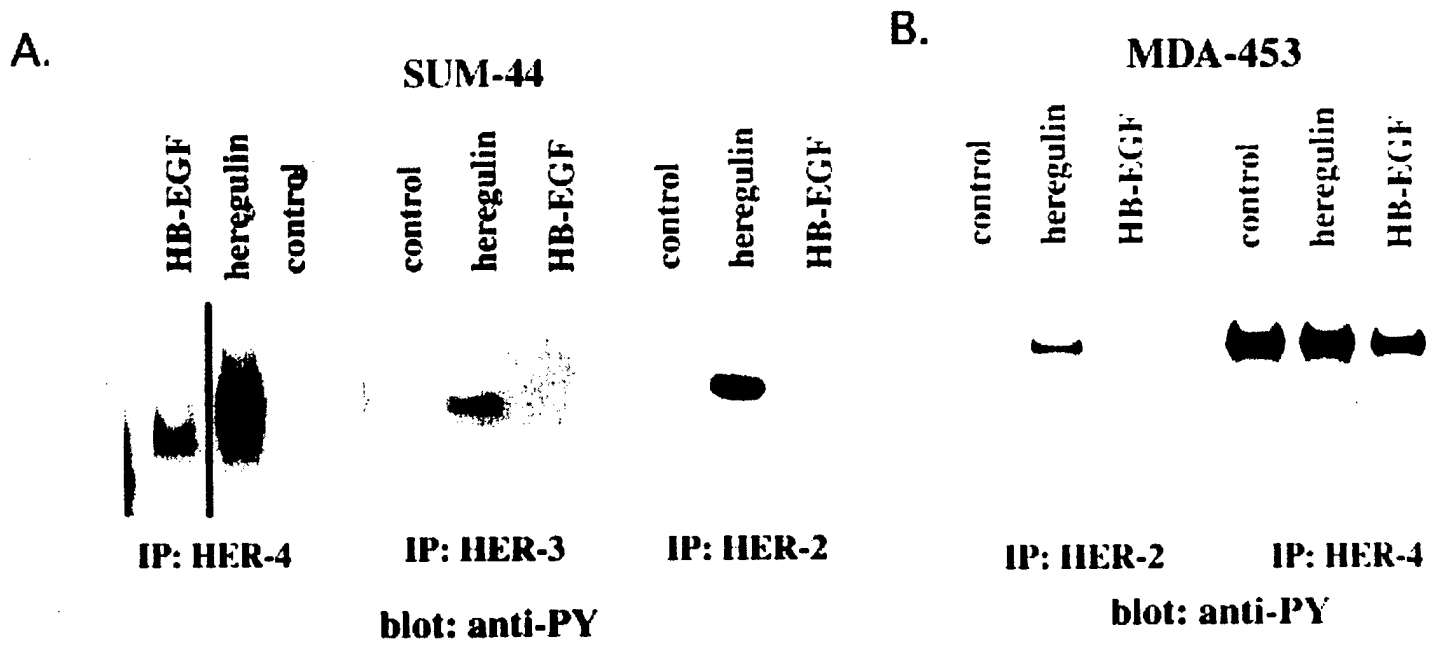


Figure 10. HER2-4 tyrosine phosphorylation in response to heregulin stimulation. SUM44 cells (A) or MDA-453 cells (B) were treated or not with 10 ng/ml heregulin B1 or 100 ng/ml HBV-EGF for 30 m inutes. Cell lysates were immunopreciptated with anti-HER2, HER3, or HER4 and immunoblotted with anti-phosphotyrosine. Heregulin induced tyrosine phosphorylation of HER2, HER3, and HER4 in SUM44 cells. Heregulin induced tyrosine phosphorylation of HER2 and HER3 in MDA453 cells, but these cells demonstrated constitutively phosphorylated HER4, which was not further induced by heregulin. HB-EGF induced tyrosine phosphorylation of only HER4 in SUM44 cells.

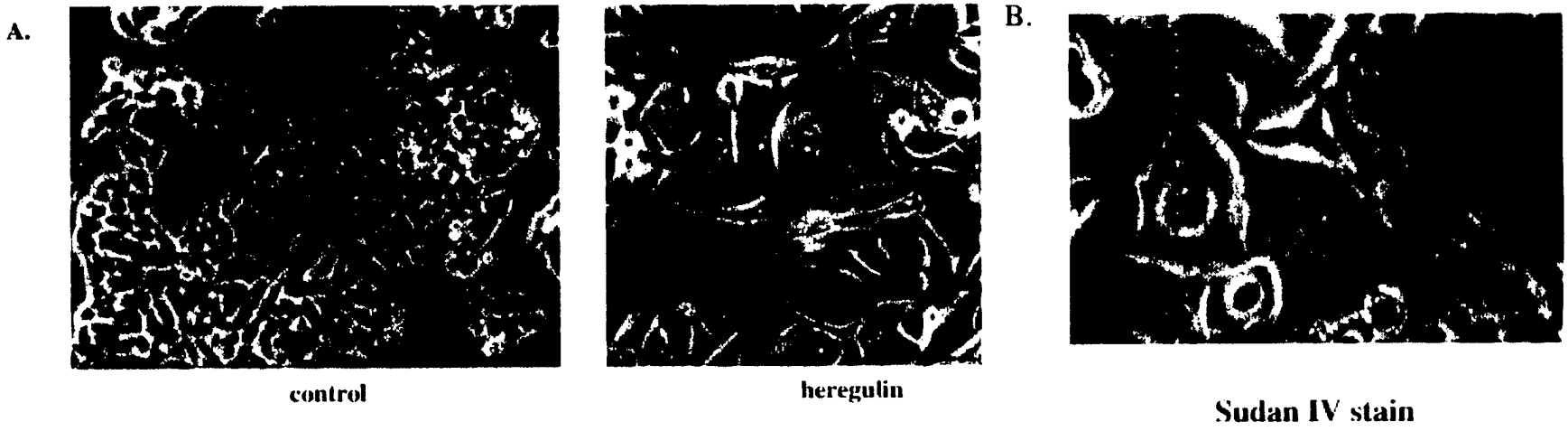


Figure 11. Differentiation changes in SUM44 cells in response to heregulin. SUM44 cells were grown in the presence or absence 10ng/ml of heregulin B1 for 1 week and photographed live (A) or after staining with Sudan IV, a neutral lipid stain (B). In the presence of heregulin, cells become larger and flattened, with prominent vacuolization. Sudan IV staining demonstrates lipid droplet formation in heregulin-treated cells.

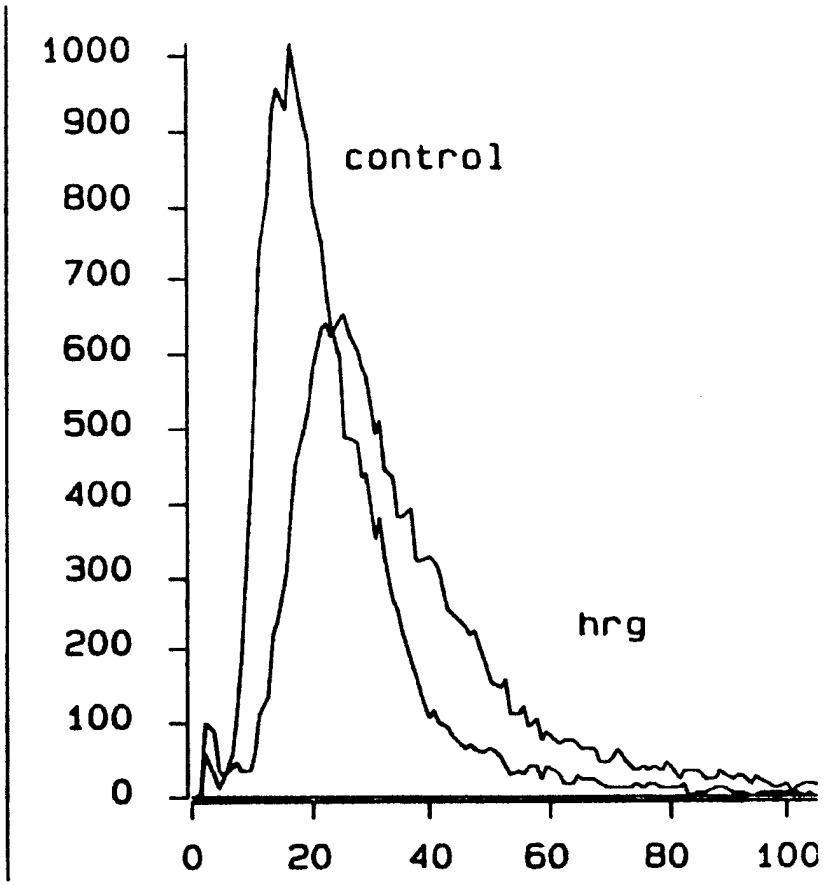


Figure 12. To quantify the extent of neutral lipid production, cells were stained with a fluorescent neutral lipid stain, Nile Red, and analyzed by FACS. Treatment with heregulin induces accumulation of neutral lipids, as evidenced by a shift of the curve toward higher intensity staining in the heregulin-treated cells.

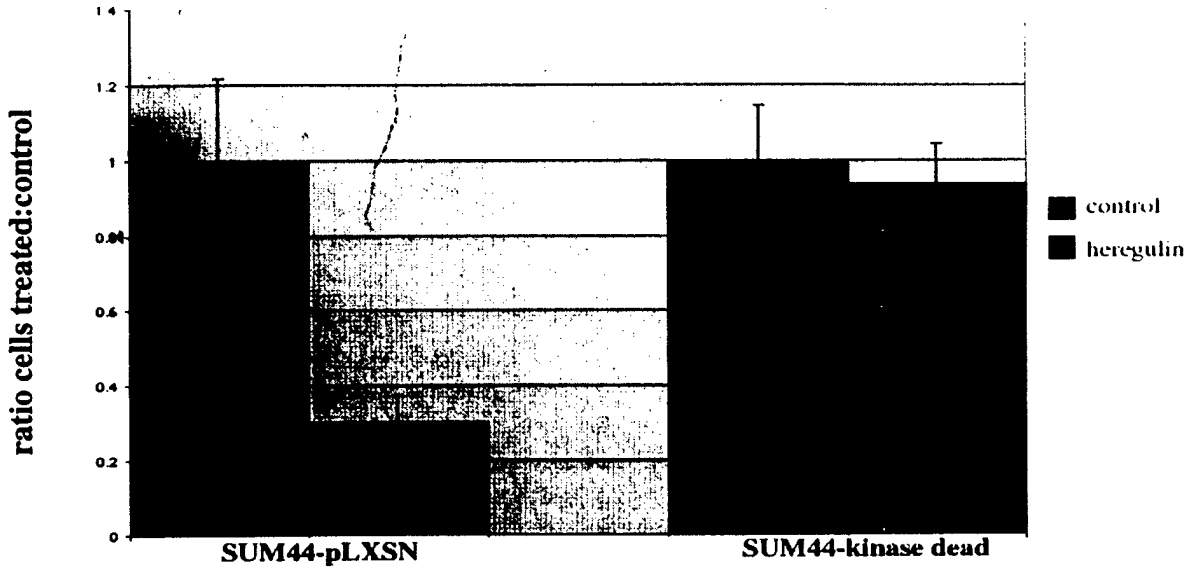


Figure 13. Manipulation of HER4 in SUM44 cells. Full-length HER4 containing a mutation in the ATP binding domain which renders it kinase dead (kdHER4) was expressed in SUM44 cells by retroviral infection. After selection in G418, expression was confirmed by RT-PCR. SUM44-kdHER4 cells or vector control cells were plated at a density of $5x10^5$ cells per well in 6-well plates and grown in the presence or absence of 10 ng/ml heregulin B1 for 3 media changes (7 days), and the number of cells counted. The ratio of number of cells grown in the presence vs. in the absence of ligand is shown. Error bars represent standard deviation of at least 3 experiments. Control cells demonstrated growth inhibition in response to heregulin, but this response was abrogated in cells expressing kinase dead HER4.

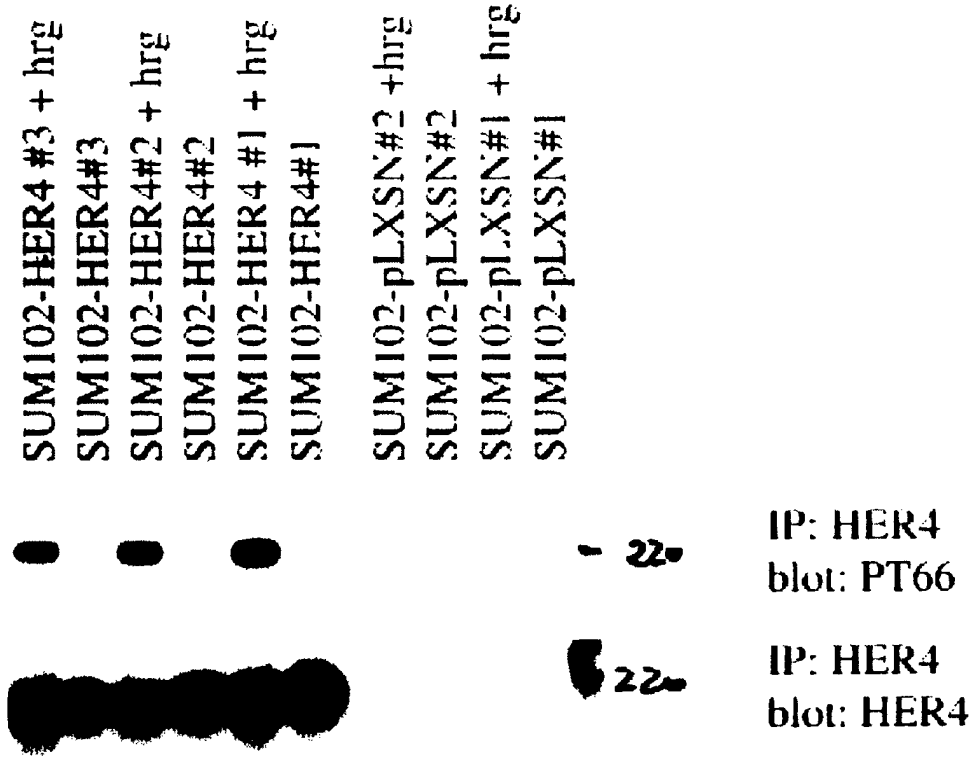


Figure 14. Stably infected SUM102 cells express HER4 that is activated by heregulin. Fu ll-length HER4 was stably expressed in SUM102 cells, a human breast cancer cell line that is HER4 negative, by retroviral infection and selection for G418 resistance. HER4 expression was confirmed by western blot using HER4 anti-serum. Vector expression was confirmed in control cells by RT-PCR of neomycin-resistant cells (data not shown). Tyrosine phosphorylation of HER4 in response to heregulin stimulation was measured by immunoprecipitation with anti-HER4 and western blot with anti-phosphotyrosine. In SUM102-HER4 lines, HER4 is not constitutively activated, but activated in response to ligand stimulation.

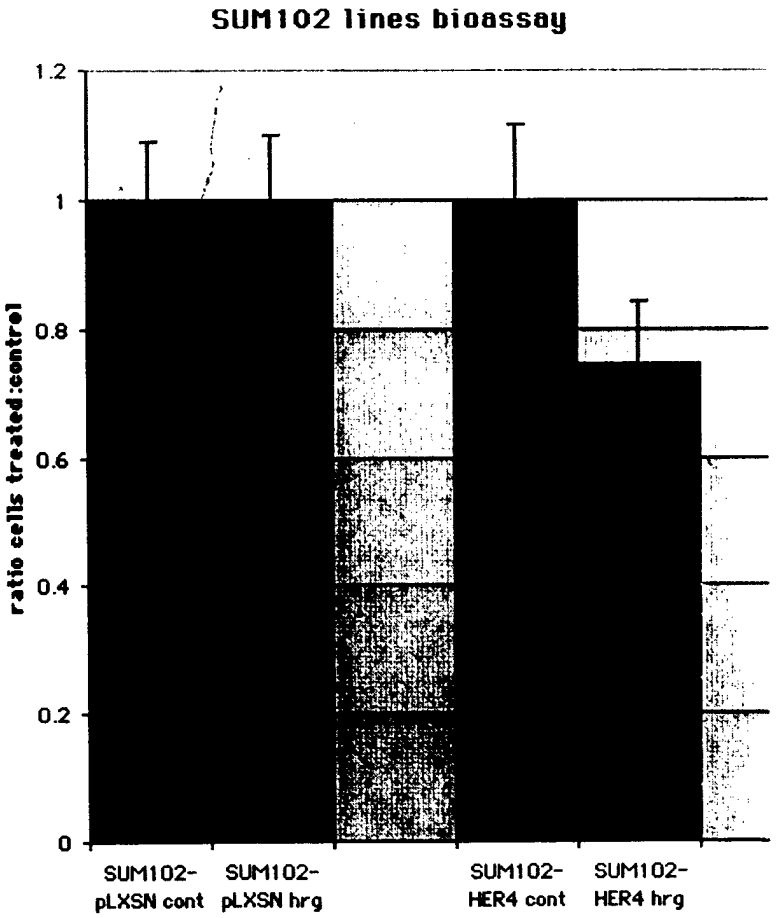


Figure 15. SUM102 anti-proliferative and differentiative response to heregulin with and without HER4. SUM102-HER4 or vector control cells were plated at a density of $5x10^5$ cells per well in 6-well plates and grown in the presence or absence of 10 ng/ml heregulin B1 for 3 media changes (7 days), and the number of cells counted. The ratio of number of cells grown in the presence vs. in the absence of ligand is shown. Error bars represent standard deviation of at least 3 experiments. SUM102 HER4 cells are growth inhibited with heregulin, comparable to SUM44 cells, while wild-type and vector control cells do not have an antiproliferative response to HER4.

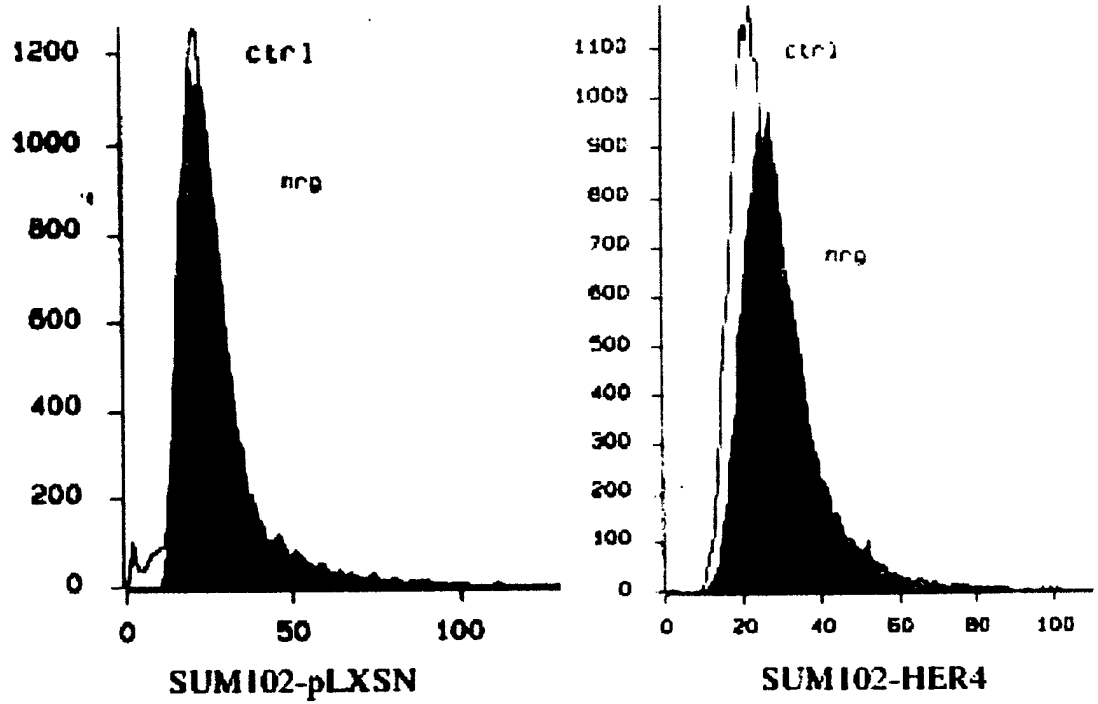


Figure 16. SUM102 cells expressing vector or HER4 were treated with 10 ng/ml heregulin for 4-6 days, and stained with Nile Red to detect neutral lipids. Instensity of staining was measured by flow cytometry, and histograms of control and heregulin-treated cells overlaid. SUM102-HER4 cells have increased neutral lipid staining when treated with heregulin, comparable to SUM44 cells, while HER4 negative control cells do not.

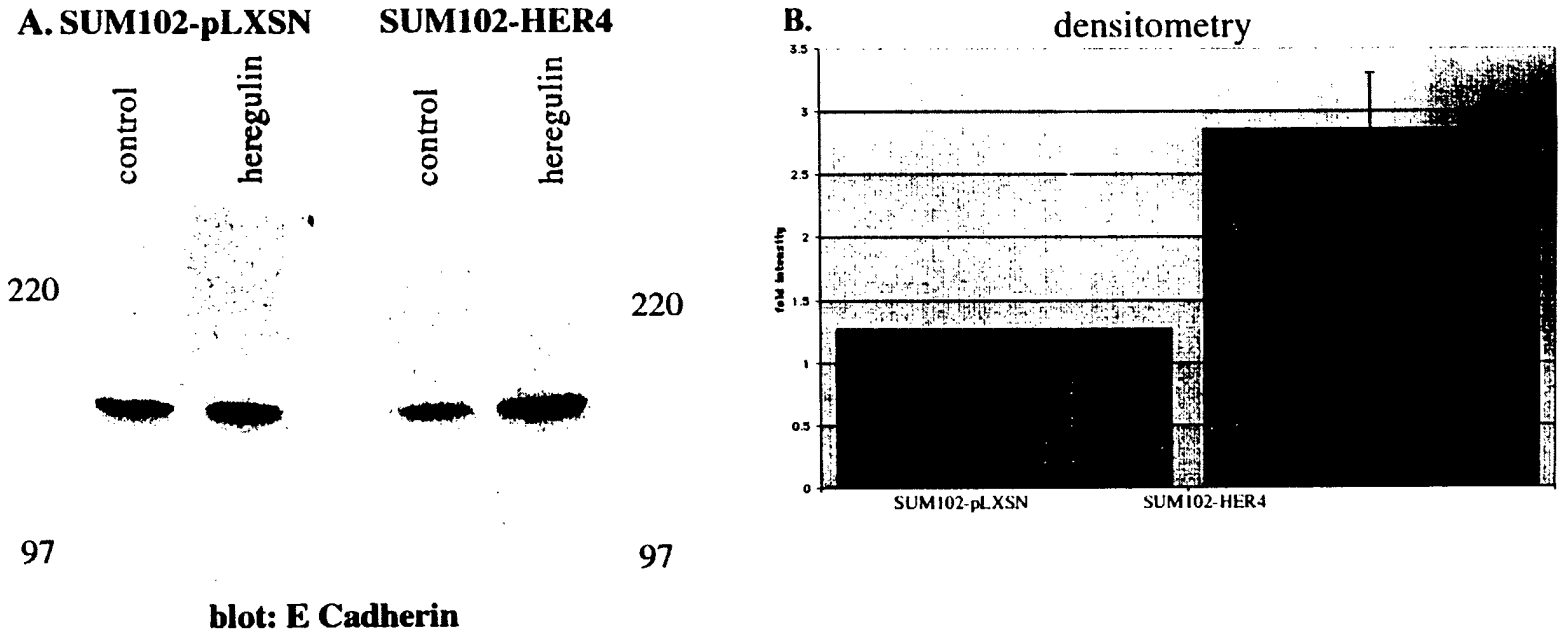


Figure 17 E-cadherin expression in SUM102-pLXSN vector control cells or SUM102-HER4 cells. (A) SUM102-pLXSN vector control cells or SUM102-HER4 cells were treated with 10 ng/ml heregulin for 4-6 days, lysed, and western blotting performed with anti-E Cadherin antibody. (B) Densitometry was performed, and standard deviation of at least 3 experiments is shown by the error bars. SUM102-HER4 but not SUM102-pLXSN demonstrated increased expression of E Cadherin in response to heregulin.

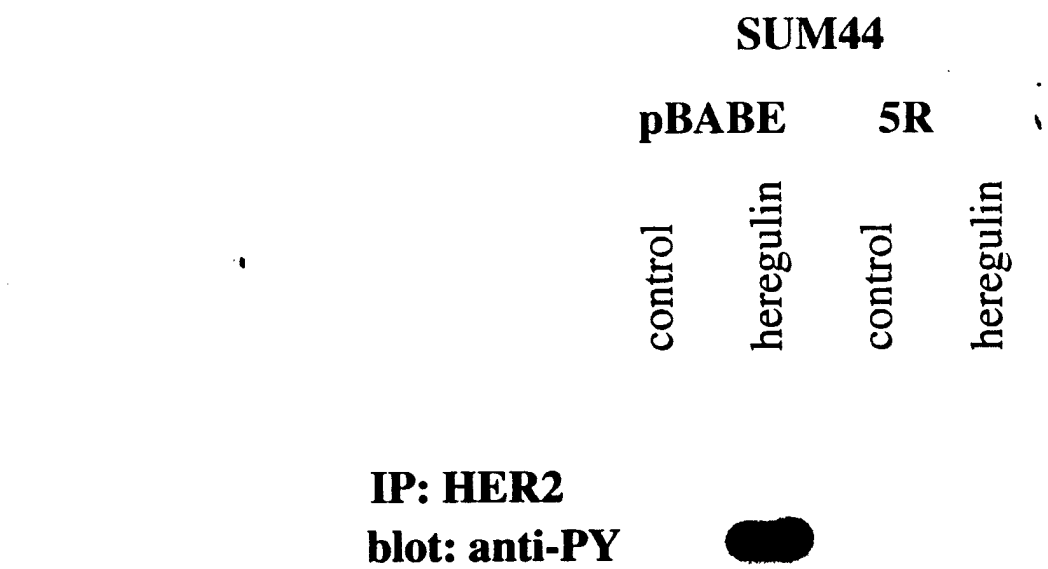


Figure 18. Effect of intracellular single chain anti-HER2 antibody expression: Abolition of HER2 signaling. SUM44 cells and SUM102-pLXSN or SUM102-HER4 cells were infected with retrovirus containing vector alone or containing the anti-HER2 endoplasmic reticulum-tagged single chain antibody 5R. After selection in puromycin, removal of HER2 from the membrane by 5R was confirmed by immunohistochemistry, demonstrating loss of HER2 membrane immunoreactivity in the 5R-containing lines (data not shown). Tyrosine phosphorylation of HER2 in response to heregulin stimulation was measured by immunoprecipitation with anti-HER2 and western blot with anti-phosphotyrosine. Cells containing the 5R construct did not have surface HER2 and did not demonstrate HER2 tyrosine phosphorylation, as opposed to cells containing vector control (shown for SUM44 cells). This demonstrates that 5R effectively abrogates HER2 signaling in these cells.

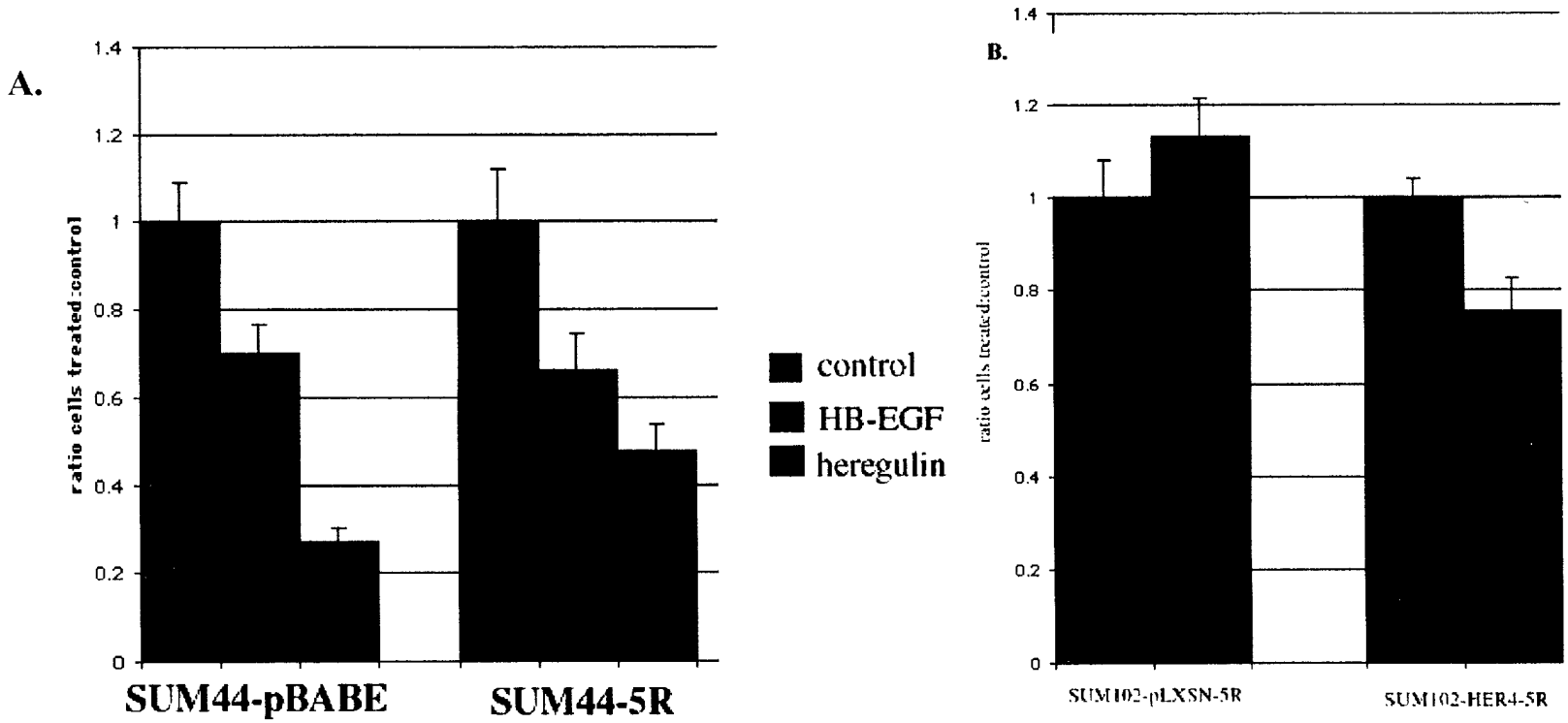
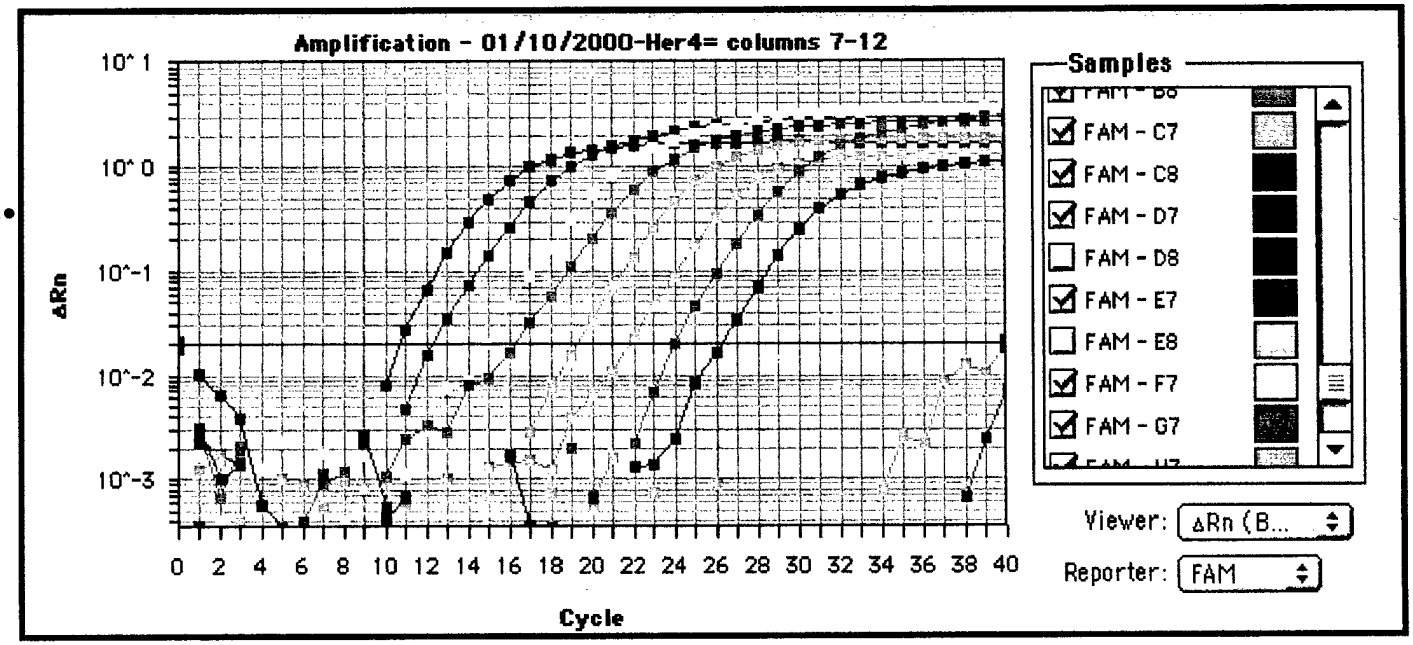


Figure 19 Anti-proliferative effect of heregulin persists in cells in which HER2 signaling is removed. (A) SUM44 cells containing vector or 5R (B) SUM102-Her4 cells or vector control cells containing 5R were plated at a density of $5x10^5$ cells per well in 6-well plates and grown in the presence or absence of 10 ng/ml heregulin B1 or 100 ng/ml HB-EGF for 3 media changes (7 days), and the number of cells counted. Error bars represent standard deviation of at least 3 experiments. Sequestration of HER2 and removal of HER2 tyrosine phosphorylation did not abrogate the antiproliferative effect of either SUM44 cells or SUM102-HER4 cells. Control SUM102-5R cells (which do not express HER4) did not demonstrate a ligand dependent anti-proliferative effect.

A.



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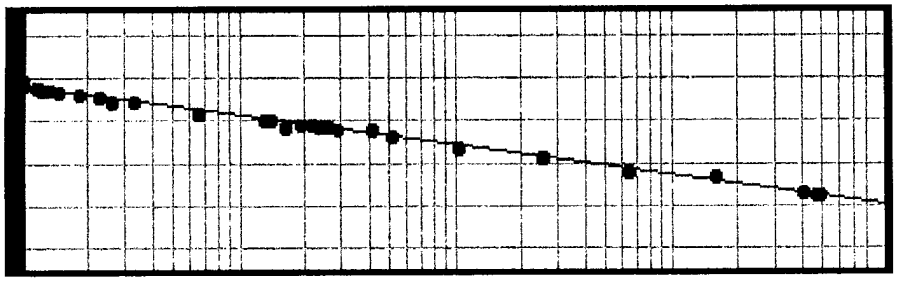

Figure 20. Amplification plot of HER4 sRNA and standard curve showing HER4 values in various breast cancer cell lines. (A) ABI 7700 analysis of in vitro transcribed HER4 mRNA. (B) Standard curve and unknowns of HER4 mRNA expression from cell lines. Black dots are standards and red dots are values from various breast cancer cell lines.

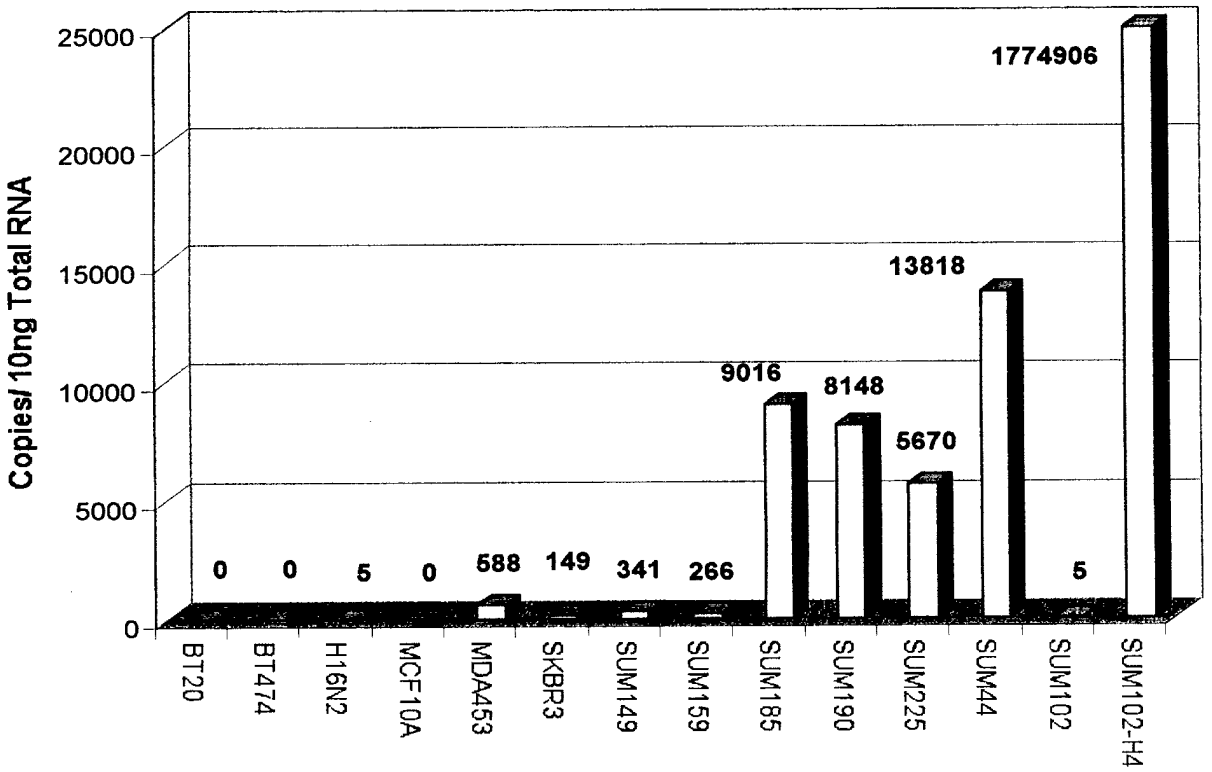

Figure 21. HER4 copies in breast cancer cell lines. Total RNA was extracted from cell lines using guanidinium isothiocyanate, treated with Dnase, and the concentration determined using Ribogreen fluorescence. Expression of HER4 was determined by real time fluorescence quantitative PCR using HER4 synthetic RNA as a positive control and absolute standard. Values indicate copies of HER4 per 10 ng of total RNA as determined by the standard curve.

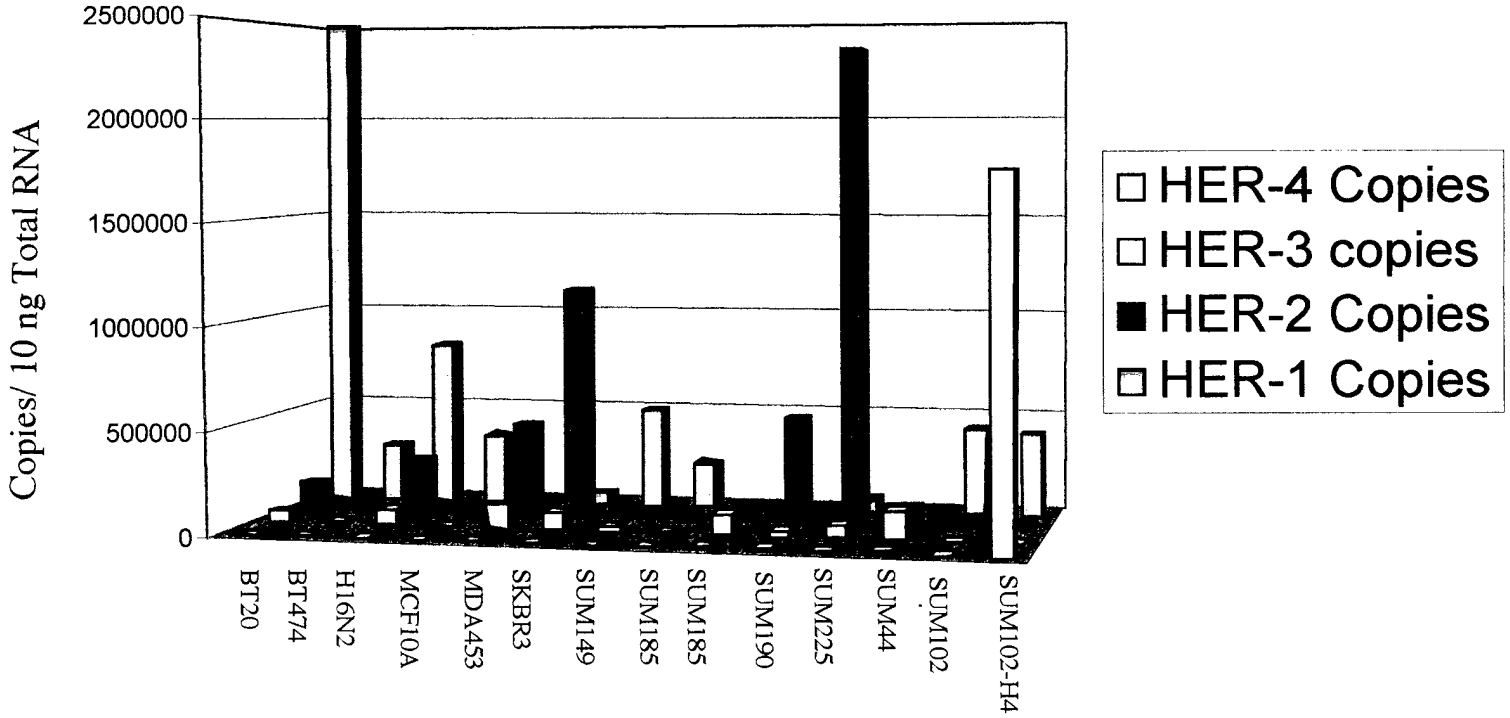

Figure 22. HER1-4 copies in breast cancer cell lines. Total RNA was extracted from cell lines using guanidinium isothiocyanate, treated with Dnase, and the concentration determined using Ribogreen fluorescence. Expression of HER1-4 was determined by real time fluorescence quantitative PCR using HER1-4 synthetic RNA as positive controls and absolute standards. Copies of HER1-4 per 10 ng of total RNA as determined by standard curves are shown.

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Appendix 1

MOLECULAR AND CELLULAR BIOLOGY, July 2001, p. 4265–4275 Vol. 21, No. 13
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.13.4265–4275.2001

HER4 Mediates Ligand-Dependent Antiproliferative and Differentiation Responses in Human Breast Cancer Cells

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Received 2 October 2000/Returned for modification 11 December 2000/Accepted 28 March 2001

The function of the epidermal growth factor receptor (EGFR) family member HER4 remains unclear because its activating ligand, heregulin, results in either proliferation or differentiation. This variable response may stem from the range of signals generated by HER4 homodimers versus heterodimeric complexes with other EGFR family members. The ratio of homo- and heterodimeric complexes may be influenced both by a cell's EGFR family member expression profile and by the ligand or even ligand isoform used. To define the role of HER4 in mediating antiproliferative and differentiation responses, human breast cancer cell lines were screened for responses to heregulin. Only cells that expressed HER4 exhibited heregulin-dependent antiproliferative responses. In-depth studies of one line, SUM44, demonstrated that the antiproliferative and differentiation responses correlated with HER4 activation and were abolished by stable expression of a kinase-inactive HER4. HB-EGF, a HER4-specific ligand in this EGFR-negative cell line, also induced an antiproliferative response. Moreover, introduction and stable expression of HER4 in HER4-negative SUM102 cells resulted in the acquisition of a heregulin-dependent antiproliferative response, associated with increases in markers of differentiation. The role of HER2 in these heregulin-dependent responses was examined through elimination of cell surface HER2 signaling by stable expression of a single-chain anti-HER2 antibody that sequestered HER2 in the endoplasmic reticulum. In the cell lines with either endogenously (SUM44) or exogenously (SUM102) expressed HER4, elimination of HER2 did not alter HER4-dependent decreases in cell growth. These results suggest that HER4 is both necessary and sufficient to trigger an antiproliferative response in human breast cancer cells.

The epidermal growth factor receptor (EGFR) family has been implicated in breast cancer pathogenesis and progression (reviewed in references 13 and 39). Aberrant expression of at least two of the family members, EGFR and HER2, has been associated with poor prognosis and differential response to therapy (21, 28, 31, 44). Recently, treatment targeted against HER2 has demonstrated clinical efficacy, emphasizing the importance of members of this receptor family in breast cancer prognosis and therapy (10).

The EGFR family consists of four known members: EGFR (HER1, erbB-1), HER2 (erbB-2), HER3 (erbB-3), and HER4 (erbB-4) (reviewed in references 13, 34, and 39). The four receptors form homodimers or heterodimers upon activation by two sets of ligands, the EGF and heregulin/neuregulin families. There are several possible hetero- and homodimeric receptor combinations, which theoretically result in differential activation of multiple downstream signal transduction pathways. Additional heterogeneity results from varying phenotypic responses, depending on cell type and the duration or intensity of downstream signaling, determined in part by differences in ligand affinity, recycling, and intracellular environment, as well as other factors that govern the turnover of receptor family members (53). Because of this complexity, our understanding of EGFR family member biology is still relatively rudimentary, despite the clinical utility of biologic modifiers of EGFR and HER2.

The EGFR family members share structural and sequence similarity; there are, however, critical differences. HER2 has no known directly binding ligand but is the favored heterodimerization partner of each ligand-bound family member (50). HER3 has no significant kinase activity, unlike the other family members, but contains multiple phosphatidylinositol 3-kinase binding motifs that are phosphorylated by a heterodimeric kinase-active partner (20, 45). HER4 is more similar to EGFR and HER2 than to HER3, but it does contain a canonical phosphatidylinositol 3-kinase binding motif (14). Unlike EGFR and HER2, which have been associated with more aggressive clinical breast cancers, in several case series HER4 expression was correlated with low proliferative index and estrogen receptor expression, suggesting that HER4 may have a different impact on breast biology and cancer.

Heregulin, or Neu differentiating factor, is a member of a complex ligand family that was initially thought to be the long-sought HER2 ligand but was ultimately shown to activate HER2 through heterodimerization after binding to HER3 or HER4 (2, 7, 23, 33, 36, 54). Heregulin was also identified as a factor that caused differentiation in MDA-MB-453 human

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breast cancer cells (11), but its biologic effect, proliferation or differentiation, differed depending upon the cell lines used during its purification, hence the two names (heregulin and Neu differentiating factor). Subsequent work by many groups has shown that heregulin is expressed as multiple isoforms (reviewed in reference 34). Heregulin α, β 1, β 2, and β 3 were cloned, and heregulin β1 was shown to cause tyrosine phosphorylation of p185 HER2 (23). Heregulin β3 is a soluble form of heregulin, while other isoforms are at least initially membrane bound (54). A second genetic locus encodes neuregulin-2, which also causes MDA-MB-453 morphologic change but with less potency and less HER2 phosphorylation (7, 8). In general, heregulin isoforms have variable potency and receptor specificity. Heregulin α and β have different effects on mouse mammary development (26). Neuregulin 2 binds to HER3 and HER4, but there is a newly discovered third gene whose product, neuregulin 3, thus far has been found to bind to HER4 alone (56). Recently, neuregulin 4 has been identified (22).

The cell-type-specific effects of heregulin-induced proliferation or differentiation may be related to the expression, activation, and level of HER2, HER3, or HER4. Because heregulin causes HER2 tyrosine phosphorylation indirectly through its binding to HER3 and HER4, the ligand could mediate its differentiative or proliferative signal singly through HER4 or through complexes containing combinations of HER2, HER3, and HER4 (33). With this complexity of potency, receptor specificity, tissue distribution, and soluble or membrane-bound isoforms, it is not surprising that different experimental results have been obtained using different cells or isoforms. The β2 isoform of heregulin caused differentiation in MDA453 cells (1). However, the β3 isoform proved to be mitogenic in the same cell line (5). Others have demonstrated a differentiation response using the β1 isoform (9, 35). In addition, the response has been shown to be concentration dependent. In AU565 and MDA-MB-453 cells a low concentration of heregulin is mitogenic, whereas a higher concentration leads to differentiation and inhibition of cell growth (2). There are also differences in response to heregulin depending on the receptor density. In a panel of human breast cancer cell lines, level of expression of HER2 correlated with response to heregulin; cells expressing low levels of HER2 had mitogenic responses to heregulin, while cells expressing high levels of HER2 had differentiation responses (12, 17, 42). Response also depends on the cell line used and the amount of serum in the medium, as expected due to heterogenous expression of receptors and ligand (27).

HER4 can also be activated by another complex family of ligands—the EGF family. Like the heregulins, there is considerable variability in receptor activation and potency. Heparin-binding EGF (HB-EGF) binds to EGFR and HER4 (15) and induces HER4 phosphorylation in MDA-MB-453 cells. Betacellulin also binds EGFR and HER4 but not other EGFR family members (5, 37). Epiregulin activates all four EGFR family members and, in MDA-MB-453 cells (which lack appreciable EGFR), causes a differentiated phenotype (25, 38, 43). Affinity labeling and competition experiments demonstrate that epiregulin binds cooperatively to HER2-HER4, but not to HER3-HER4, heterodimers and directly binds EGFR and HER4.

Because HER4 appears to be associated with better prognostic features and can be activated by differentiation-inducing ligands, we attempted to clarify the role of HER4 by asking whether HER4 alone was necessary and sufficient to transmit an antiproliferative signal. We determined that HER4 activation by a member of the heregulin or the EGF family could transmit an antiproliferative response, and that expression of HER4 in a HER4-negative cell line was sufficient to confer an antiproliferative response. Perhaps most intriguingly, elimination of HER2 signaling did not abolish HER4-dependent antiproliferative responses in at least two distinct cell lines.

MATERIALS AND METHODS

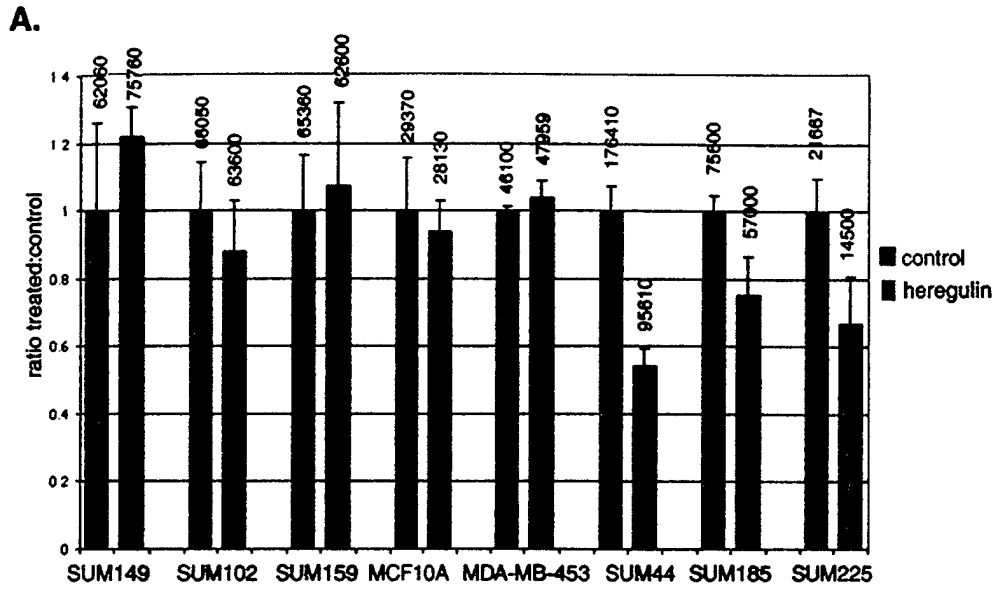
Cell lines, tissue culture, and antibodies. SUM44 and SUM102 cells were grown in serum-free growth factor-defined media as previously described (16, 41). SUM102 cells were derived from a microinvasive primary breast tumor, whereas SUM44 cells were derived from a metastatic pleural effusion. MDA-MB-453 cells were obtained from the American Type Culture Collection and were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. All cells were grown in a humidified incubator at 37°C with 10% CO_2 and subcultured weekly, and the medium was changed three times per week. All tissue medium reagents were obtained from Sigma, except for fetal bovine serum and insulin, which were obtained from Gibco BRL.

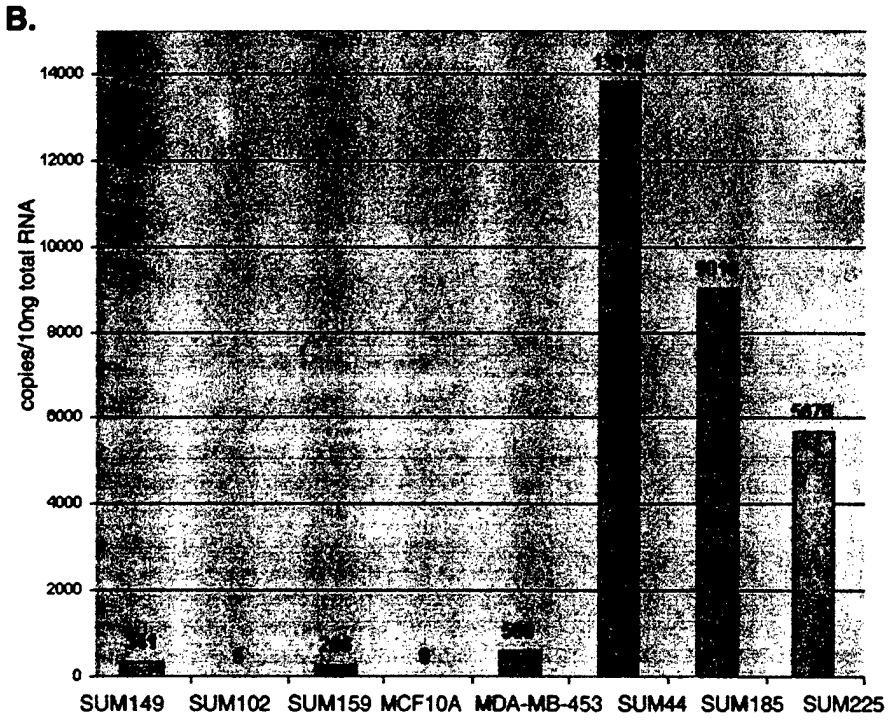
Proliferation assays. Cells were plated into six-well plates at a density of 5×10^4 to 5×10^5 cells per well and grown in the appropriate medium with or without recombinant heregulin β1 (gift from Amgen) or HB-EGF (R&D) for 6 to 7 days, or three medium changes. Cells were trypsinized and counted with a hemocytometer.

Quantitative PCR. Total RNA was isolated using the guanidinium isothiocyanate-based RNeasy kit (Qiagen) and was treated with RNAse-free DNAse (Ambion) to prevent nonspecific priming of the PCRs. HER4-specific 5′-3′ oligonucleotides and an intervening fluorescent dye-labeled probe were designed using Primer Express software (ABI/Perkin Elmer). The nonextendable HER4 probe was synthesized and labeled with 5′ FAM (6-carboxyfluorescein) reporter and 3′ TAMRA (6-carboxy-tetramethyl-rhodamine) quencher dyes (Integrated DNA Technologies), followed by high-pressure liquid chromatography purification. Real-time fluorescence quantitative PCR was performed with the ABI PRISM 7700 (PE Bio). Full-length HER4 mRNA was in vitro transcribed using MEGAscript (Ambion) and used as a positive control and absolute quantitation standard for the assays. Similarly transcribed constructs for HER1, HER2, and HER3 were used as negative controls. Amplifications of twofold serial dilutions of full-length HER4 RNA were used to construct standard linear curves that permit us to routinely and accurately measure from 200 copies to 90 million template copies of HER4 mRNA. Ten nanograms of total RNA isolated from the cell lines was assayed in triplicate for HER4 expression levels.

Immunoprecipitation and immunoblot analysis. Cells were washed with cold phosphate-buffered saline and lysed in lysis buffer containing 20 mM HEPES (pH 7.3), 50 mM sodium fluoride, 10% glycerol, 1% Triton X-100, 5 mM EDTA, and 0.5 M NaCl supplemented with the tyrosine phosphatase inhibitor sodium orthovanadate (1 mM) and the protease inhibitors aprotinin(6 μg/ml) and leupeptin(10 μg ml). Nuclei and insoluble material were removed by centrifugation at 13,000 × g for 10 min at 4°C. Receptor proteins were precipitated with various antibodies [HER2, clone 9G6.10, mouse monoclonal antibody (Neomarkers, Inc.); HER3, (c-17)G, goat polyclonal antibody (Santa Cruz); HER4, polyclonal rabbit antisera raised against recombinant gluthathione *S*-transferase fusion protein containing the C-terminal 80 amino acids of HER4] and protein A/G or protein A agarose beads (Santa Cruz) for 3 h at 4°C. Immune complexes were washed three times with lysis buffer and denatured in sodium dodecyl sulfate sample buffer. Protein samples were separated on a sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis gel and were electrophoretically transferred to a Sequi-blot polyvinylidene difluoride membrane (Bio-Rad). After blocking with 3% cold fish gelatin (Sigma), the membrane was probed overnight at 4°C with antiphosphotyrosine antibody (RC20; Transduction Laboratories), washed three times with Tris-buffered saline–Tween, and detected with an enhanced chemiluminescence detection kit (Amersham Life Sciences).

Neutral lipid detection. Cells were grown on glass coverslips in appropriate media with or without heregulin or HB-EGF for 1 week and then fixed with 10% neutral buffered formalin for 10 min. After a 60% isopropyl alcohol rinse, they were stained with Sudan IV solution (10 g of Sudan IV, 500 ml of acetone, 500 ml of 70% ethyl alcohol) for 4 min, followed by 60% isopropyl alcohol and distilled water rinses. Cells were then stained with Gill's hematoxylin (Fisher Scientific) for 1 min, rinsed in distilled water, and then stained with lithium



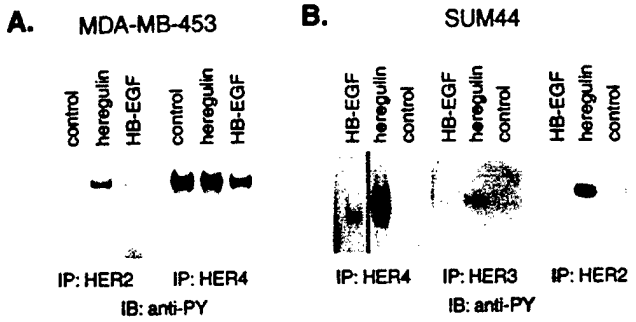


C.

Copies (10^3) per 10ng total RNA

	SUM149	SUM102	SUM159	MCF10A	MDA-MB-453	SUM44	SUM185	SUM225
HER1	494	431	228	349	8	0	3	62
HER2	31	23	42	42	455	95	69	235
HER3	22	0	0	5	114	139	104	63

FIG. 1. (A) Proliferative response of human breast cancer cell lines to heregulin. Cells were plated at a density of 5×10^4 to 5×10^5 cells per well, depending on plating efficiency and growth rate, in six-well plates and grown in the presence or absence of 10 ng of heregulin β1 per ml for three medium changes (7 days; approximately three doublings), and the number of cells was counted. The ratio of number of cells grown in the presence versus the absence of heregulin is shown, with the number of cells (average of three experiments) listed at the top of each column. Error bars represent standard deviations of at least three experiments. SUM185 ($P = 0.03$), SUM225 ($P = 0.03$), and SUM44 ($P = 0.0009$) cells demonstrated a statistically significant (by Student's t test) heregulin-dependent antiproliferative effect, with the effect in SUM44 cells being most pronounced. (B) HER4 mRNA levels as determined by quantitative PCR. Total RNA was extracted and reverse transcribed, and quantitative PCR was performed with the ABI PRISM 7700 using HER4-specific fluorescence-labeled oligonucleotide probes, as described in Materials and Methods. (C) HER1, -2, and -3 mRNA levels as determined by quantitative PCR. Quantitative PCR was performed using HER1-, HER2-, and HER3-specific probes as described above. The abundance of message of the other EGFR family members is usually much higher than that of HER4.



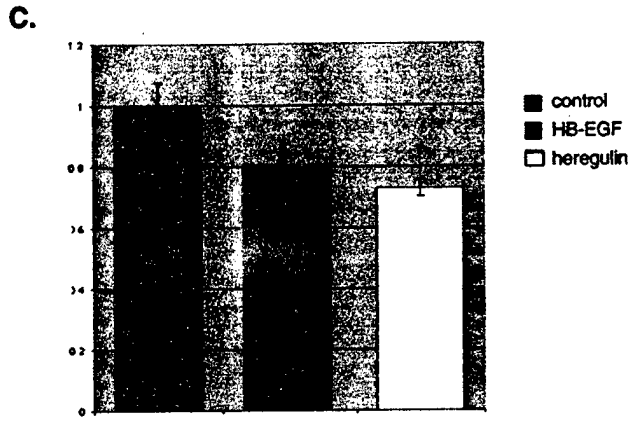


FIG. 2. Receptor tyrosine phosphorylation in response to heregulin stimulation. Cells were treated with 10 ng of heregulin β1 or 100 ng of HB-EGF per ml for 30 min or left untreated. Cell lysates were immunoprecipitated (IP) with antibodies to HER2, HER3, or HER4 and immunoblotted with antiphosphotyrosine (anti-PY) antibody RC20. (A) Heregulin induced tyrosine phosphorylation of HER2 in MDA-MB-453 cells, but these cells demonstrated constitutively phosphorylated HER4, which was not further induced by heregulin. (B) Heregulin induced tyrosine phosphorylation of HER2, HER3, and HER4 but HB-EGF induced tyrosine phosphorylation of only HER4 in SUM44 cells. (C) Antiproliferative response to HB-EGF. SUM44 cells were plated at a density of 5×10^5 cells per well in six-well plates and grown in the presence or absence of 10 ng of heregulin B1 or 100 ng of HB-EGF per ml for three medium changes (7 days), and the number of cells was counted. The ratio of number of cells grown in the presence versus the absence of ligand is shown. Error bars represent standard deviations of at least three experiments. Like heregulin, HB-EGF caused a significant antiproliferative effect, although the effect of HB-EGF was not as great as that of heregulin.

carbonate (47 g of lithium carbonate, 3,500 ml of distilled water) till blue (about 30 s). After thorough rinsing in distilled water, slides were mounted in Aquamount for direct microscopic visualization of red-staining lipid droplets. Alternatively, staining of neutral lipid droplets in the cellular cytoplasm was done as described previously (40). In brief, the cells suspended in phosphate-buffered saline (10^6 cells/ml) were incubated for 5 min with Nile red (final concentration, 100 ng/ml) at room temperature. The yellow fluorescence of Nile red-stained neutral lipids droplets was analyzed with a FACScan (Becton Dickinson) using linear amplifiers.

cDNA constructs and clones. Full-length human HER4 cDNA was created from three PCR fragments amplified from MDA-MB-453 cells. The fragments were recombined into the pLXSN retroviral vector (29), and the resulting full-length cDNA was sequenced in its entirety. The kinase-dead HER4 construct was created by site-directed mutagenesis, changing lysine to alanine in the 751 position and abolishing the ability to bind ATP. The construct was entirely sequenced and cloned into pLXSN. The 5R construct is a HER2 single-chain antibody with an endoplasmic reticulum (ER)-targeting sequence cloned into the pBABEpuro vector (19).

Creation of cell lines stably expressing introduced constructs. For production of retrovirus using the above cDNAs in pLXSN, the amphotropic packaging cell line PA317 was plated at 5×10^5 cells per 60-mm dish and then transfected with 20 μg of retroviral DNA using 2 M $CaCl_2$ precipitation as previously described (32). Viral supernatants were collected after 60 h of incubation, the last 48 h at 37°C with addition of sodium butyrate as described previously (30). Viral supernatants were filtered through a 0.45-μm-pore-size syringe filter, and 1 ml of viral supernatant was added with 8 μg of Polybrene per ml to recipient cells which had been plated at 7×10^5 cells per 100-mm dish the day before. After 48 h of incubation, cells were placed in medium containing G418 (0.3 mg/ml for SUM102 and 0.5 mg/ml for SUM44). G418-resistant, puromycin-resistant, or G418- and puromycin-resistant cells were pooled, and expression of the cDNA product was confirmed by Western blotting or reverse transcription-PCR (RT-PCR).

RESULTS

Proliferative response of human breast cancer cell lines to heregulin. Heregulin has been shown to cause alternatively a mitogenic or an antimitogenic effect under various experimental conditions. To determine the range of this effect, and to select appropriate cell lines for study of the differentiative effects of heregulin, we characterized the proliferative response to heregulin of a panel of human breast cancer cell lines, many of which had not previously been evaluated for their heregulin response. The cell lines differ in their EGFR family member expression (Fig. 1B and C) and their exogenous ligand requirements. Many grow under growth factor-defined, serum-free conditions, allowing evaluation of the effect of heregulin without the confounding factor of undefined serum growth factors and without subjecting the cells to serum starvation. Three cell lines demonstrated a significant growth inhibitory response to heregulin: SUM44, SUM185, and SUM225 (Fig. 1A). The MDA-MB-453 cells obtained from the American Type Culture Collection were only minimally responsive to heregulin and in fact grew slowly under the conditions tested. As discussed below, they appeared to have constitutive activation of HER4, which precluded their use for evaluation of ligand dependence. MCF10A cells, which have been shown to have a proliferative response to heregulin when grown in serum-containing medium or when starved of insulin or EGF, did not demonstrate a proliferative effect when heregulin was added to defined medium containing EGF and insulin.

To correlate the antiproliferative response with HER4 expression, mRNA levels from these cell lines were evaluated by quantitative PCR using the ABI PRISM 7700 (Fig. 1B and C). The three cell lines that demonstrated an antiproliferative response to heregulin all expressed HER4, while the cell lines that lacked an antiproliferative response to heregulin did not, or expressed very low levels. Thus, HER4 expression correlated with an antiproliferative response to heregulin.

Receptor tyrosine phosphorylation in response to heregulin stimulation. Previous work (1) led us to examine heregulin-dependent tyrosine phosphorylation of the EGFR family members in MDA-MB-435 cells, which have a high level of HER2 expression (Fig. 1C) and modest HER4 mRNA levels as measured by quantitative PCR. Despite low levels of HER4 message, substantial, constitutive HER4 tyrosine phosphorylation was observed, which was not further increased by heregulin treatment (Fig. 2A). In contrast, the low-level constitutive HER2 tyrosine phosphorylation was further induced by heregu-

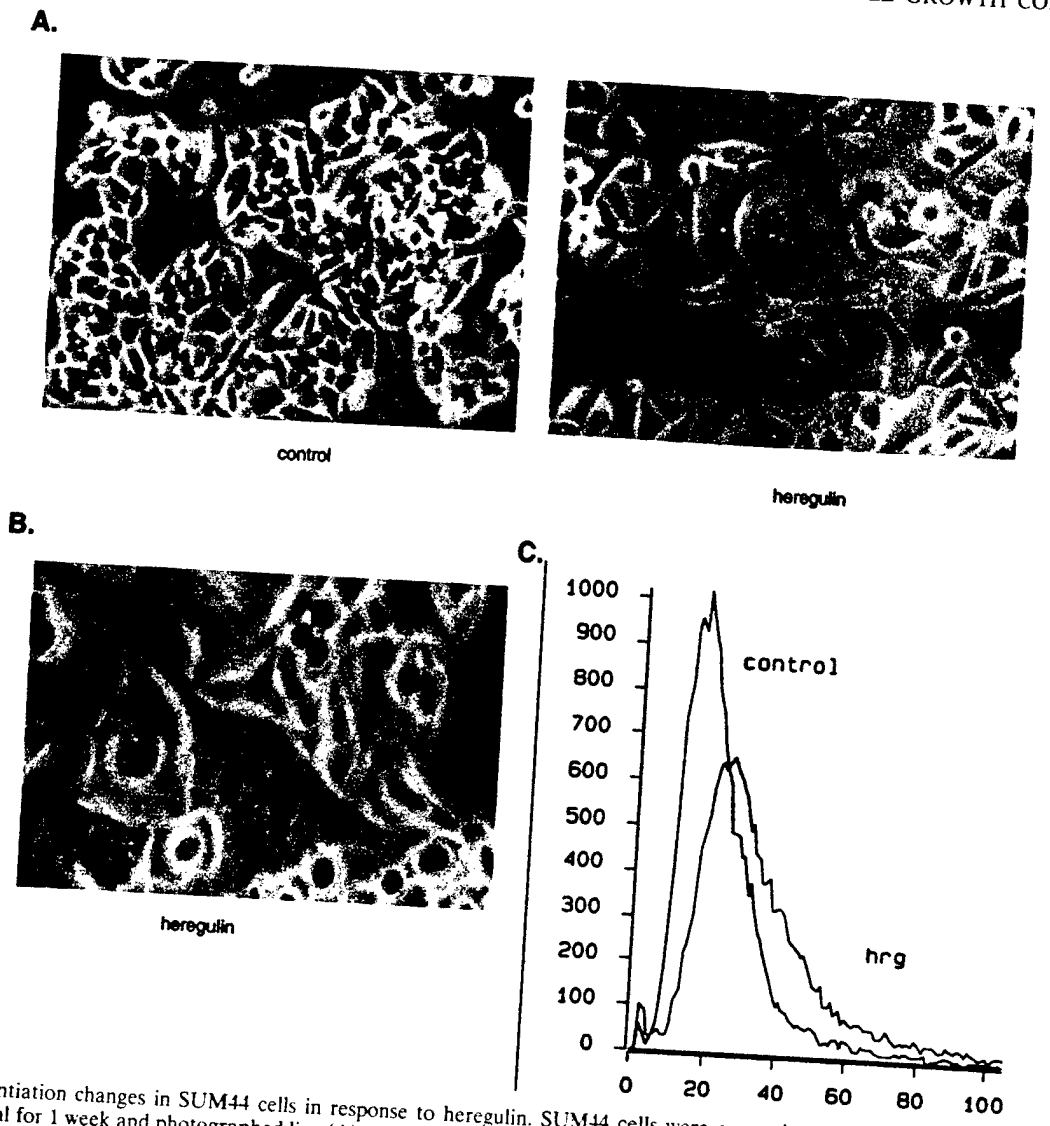


FIG. 3. Differentiation changes in SUM44 cells in response to heregulin. SUM44 cells were grown in the presence or absence of 10 ng of heregulin β1 per ml for 1 week and photographed live (A) or after staining with Sudan IV, a neutral lipid stain (B). In the presence of heregulin, cells become larger and flattened, with prominent vacuolization. Sudan IV staining demonstrates lipid droplet formation in heregulin-treated cells. (C) To quantify the extent of neutral lipid production, cells were stained with a fluorescent neutral lipid stain, Nile red, and analyzed by FACS. Treatment with heregulin induces accumulation of neutral lipids, as evidenced by a shift of the curve toward higher-intensity staining in the heregulin (hrg)-treated cells.

lin. Thus, any antiproliferative effect mediated by HER4 was already near the maximum, and in fact this clone of MDA-MB-453 cells proliferates slowly even in the absence of heregulin, displaying the flattened morphology with prominent vacuolization and the high cytoplasm-to-nucleus ratio typical of the differentiated phenotype described for MDA-MB-453 cells. Therefore, despite clear induction of HER2 phosphorylation by heregulin, there was no positive or negative proliferative change, and this clone was not useful for ligand-dependent studies.

In contrast, SUM44 cells demonstrated a consistent antiproliferative response to heregulin. Without heregulin treatment there was no HER2 or HER4 activation (Fig. 2B). As anticipated, since it is a ligand for both HER3 and HER4 that can also dimerize with and activate HER2, heregulin induced tyrosine phosphorylation of HER2, HER3, and HER4.

HER4 tyrosine phosphorylation and antiproliferative response to HB-EGF. Since heregulin induces HER2, HER3, and HER4 tyrosine phosphorylation, any or all could be responsible for the antiproliferative response. Therefore, the effect of a ligand that would bind specifically to HER4 was examined. HB-EGF binds to EGFR and HER4 but not directly to HER2 or HER3 (15). As anticipated, when SUM44 cells were treated with HB-EGF, HER4 became tyrosine phosphorylated, but, in contrast to results with heregulin, HER2 and HER3 were not (Fig. 2B). SUM44 cells do not express EGFR. HB-EGF-induced HER4 tyrosine phosphorylation was not as robust as that resulting from heregulin stimulation. The consequence of HB-EGF-dependent HER4 tyrosine phosphorylation in SUM44 cells was antiproliferative, although to a lesser degree than heregulin (Fig. 2C). The attenuated antiproliferative effect of HB-EGF correlated with the lower levels

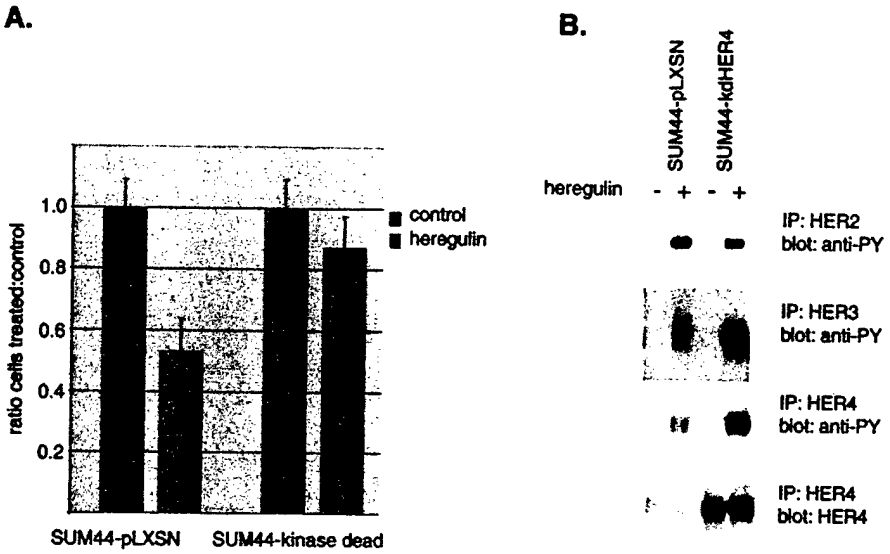


FIG. 4. (A) Introduction of a kinase-dead HER4 construct in SUM44 cells. Full-length HER4 containing a mutation in the ATP binding domain which renders it kinase dead (kdHER4) was expressed in SUM44 cells after retroviral infection and selection with G418 (expression was confirmed by RT-PCR). SUM44-kdHER4 cells or vector control cells were plated at a density of 10^5 cells per well in six-well plates and grown in the presence or absence of 10 ng of heregulin β1 per ml for three medium changes (7 days), and the number of cells was counted. Error bars represent standard deviations of at least three experiments. Control cells demonstrated significant growth inhibition in response to heregulin, but this response was attenuated in cells expressing kinase-dead HER4. (B) Ligand-induced receptor phosphorylation in vector control cells or cells expressing kdHER4. Cells were treated with 10 ng of heregulin β1 per ml for 10 min, immunoprecipitated (IP) with HER2, HER3, or HER4, electrophoresed, and blotted with antiphosphotyrosine (anti-PY). Expression of kinase-dead HER4 did not affect HER2 or HER3 phosphorylation. There was an apparent increase in HER4 phosphorylation, presumably due to endogenous phosphorylation by heterodimeric partners of the kinase-dead receptor, which is expressed at high levels.

of HER4 tyrosine phosphorylation (Fig. 2B). Thus, activation of HER4 alone correlates with an antiproliferative effect in response to HER4 ligands. In MDA-MB-453 cells, HB-EGF did not induce HER2 or HER4 tyrosine phosphorylation above the baseline constitutive activation (Fig. 2B) and did not slow growth of these cells (data not shown).

Differentiation in response to HER4 activation. Decreased proliferation is one of the phenotypic changes that occur with differentiation of human breast cancer cells, but decreased proliferation may occur without differentiation. Therefore, we looked to see whether other phenotypic changes occurred with heregulin or HB-EGF stimulation. With differentiation, breast epithelial cells change morphology, becoming more flattened with higher cytoplasm-to-nucleus ratios. They produce milk proteins, which in human cells can be measured by detecting an increase in neutral lipids. SUM44 cells were treated with heregulin and examined for morphologic changes and neutral lipid production as measured by Sudan IV staining. Over a 2- to 6-day heregulin treatment, a proportion of the SUM44 cells underwent clear morphologic changes consistent with differentiation (Fig. 3A). These and other cells without such significant morphologic changes produced neutral lipid droplets (Fig. 3B). In order to better evaluate the percentage of cells that underwent these differentiative changes, we quantified neutral lipid-producing cells by fluorescence-activated cell sorting (FACS) analysis using Nile red (Figure 3C). Heregulin clearly induced morphologic changes and neutral lipid production in SUM44 cells.

Attenuation of antiproliferative response to heregulin by expression of kinase-inactive HER4. Heregulin causes tyrosine phosphorylation of HER2 and HER3 as well as HER4 in SUM44 cells and induces differentiation changes. HB-EGF induces tyrosine phosphorylation of only HER4 in these cells and induces antiproliferative changes, suggesting that HER4 alone is responsible for transmitting the antiproliferative signal seen in response to both ligands. To further support the role of HER4 in transmitting an antiproliferative signal, we attempted to block the antiproliferative response to heregulin by interfering with HER4 activation. A kinase-inactive HER4 construct (kdHER4) that in other receptor contexts acts as a dominant negative was created by site-directed mutagenesis and introduced into SUM44 cells by retroviral infection. Selection of kdHER4- or vector-expressing cells was performed with the antibiotic G418. Cells expressing kdHER4 demonstrated increased proliferation compared with vector control cells, suggesting that kdHER4 was counteracting a growth inhibitory signal. In addition, expression of kinase-dead HER4 (but not vector) in SUM44 cells blocked the heregulin-dependent antiproliferative response (Fig. 4A). The effects of kinase-dead HER4 expression on HER2, HER3, and HER4 tyrosine phosphorylation are shown in Fig. 4B. Expression of kinase-dead HER4 did not interfere with ligand-induced HER2 or HER3 tyrosine phosphorylation. There was an apparent increase in HER4 phosphorylation, presumably due to phosphorylation of the kinase-dead receptor, which is expressed at high levels. This may result from HER2-kdHER4 heterodimers, with the HER2 providing the kinase, as occurs with EGF-dependent EGFR tyrosine phosphorylation of kinase-dead HER2. Regardless, it is clear that heregulin-dependent HER2, HER3, and HER4 tyrosine phosphorylation is insuffi-

cient to send the full HER4 signal in cells overexpressing expressing kdHER4; i.e., the antiproliferation response is attenuated. The explanation for attenuation of the HER4 signal presumably lies in the lack of specific tyrosine phosphorylation sites on HER4 phosphorylated by HER2 or, perhaps more intriguingly, the absence of activated HER4 kinase domain-engaging specific substrates (even soluble non-SH2 domain-containing substrates) that trigger the antiproliferation signal; we are currently investigating these hypotheses.

Expression of HER4 in HER4-negative cells: acquisition of antiproliferative and differentiation capability. It is possible that some unique characteristic of SUM44 cells resulted in the detection of a HER4-dependent antiproliferative response. Therefore, a second model cell system was sought. SUM102 is a primary human breast cancer cell line that does not demonstrate a proliferative or antiproliferative response to heregulin (Fig. 1A), nor does it exhibit heregulin-dependent differentiation (not shown). SUM102 cells do not express HER4 (Fig. 1B). Therefore, to determine whether expression of HER4 in a HER4-negative cell line would be sufficient to induce an antiproliferative and/or differentiation response to heregulin. SUM102 cells were infected with retrovirus containing full-length HER4 or vector alone and selected for neomycin resistance. The resistant colonies grew slowly but yielded several lines. Vector-infected control cells do not express HER4, while SUM102-HER4 lines stably express HER4 that is tyrosine phosphorylated in response to heregulin (Fig. 5). EGFR phosphorylation in response to EGF is unaffected by HER4 expression. SUM102 cells express very low levels of HER2 (Fig. 1C). which is not appreciably phosphorylated in response to heregulin whether or not HER4 is expressed. SUM102 cells do not express HER3.

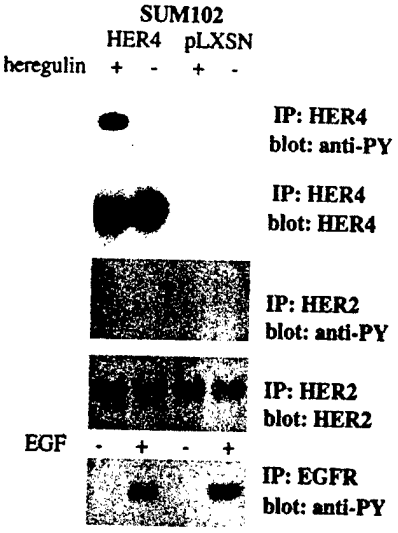


FIG. 5. Stably infected SUM102 cells express HER4 that is activated by heregulin. Full-length HER4 was stably expressed in SUM102 cells, a HER4-negative human breast cancer cell line, by retroviral infection and selection for G418 resistance. HER4 expression was confirmed by Western blotting using HER4 antiserum. Vector expression was confirmed in control cells by RT-PCR of neomycin-resistant cells (data not shown). Tyrosine phosphorylation of HER4 and HER2 in response to heregulin stimulation was measured by immunoprecipitation (IP) with antibody to HER4 or HER2 and Western blotting with antiphosphotyrosine (anti-PY). Phosphorylation of EGFR in response to EGF stimulation was similarly examined. In SUM102-HER4 lines, HER4 is not constitutively activated but is activated in response to ligand. There is no appreciable phosphorylation of HER2 in either HER4-expressing or wild-type cells, and EGFR phosphorylation in response to EGF is not altered by HER4 expression.

While neither parental SUM102 cells nor SUM102-pLXSN vector control cells demonstrated an antiproliferative or differentiation response to heregulin, SUM102-HER4 exhibited slowed growth in response to heregulin (Fig. 6A). In addition. SUM102-HER4 cells demonstrated increased neutral lipid production when treated with heregulin, while the parental SUM102 cells (data not shown) and SUM102-pLXSN control cells (Fig. 6B) did not. Thus, expression of HER4 provided SUM102 cells with both antiproliferative and differentiative responses to heregulin, suggesting that HER4 is essential for the differentiation response.

To further confirm that SUM102-HER4 cells were undergoing differentiation changes upon heregulin stimulation, we evaluated the expression of E cadherin, whose expression has been correlated with differentiation changes in a number of systems (reviewed in reference 51). Heregulin induced a 2.5-fold increase in expression of E cadherin in SUM102-HER4 cells but not in control cells (Fig. 6C), and this was quantified by densitometry (Fig. 6D). Thus. heregulin induces an antiproliferative response only in SUM102 cells that express HER4. The antiproliferative response is paralleled by differentiation changes, including neutral lipid production and increased E cadherin expression.

Removal of HER2 does not abolish the heregulin-dependent antiproliferative response. Our results in both cell lines suggested that HER4 plays a necessary role in mediating an antiproliferative and differentiation signal, but they do not answer a central question; does HER2 contribute to this response? To determine this, the capacity for HER2 signaling was removed from both SUM44 and SUM102-HER4 cells by abolishing HER2 cell surface expression. This was accomplished by sequestering HER2 in the ER by expressing single-chain anti-HER2 antibody containing an ER-targeting sequence (19). This cDNA construct, 5R, was introduced into cells after having been packaged as an amphotrophic retrovirus. Selection of infected SUM102-pLXSN, SUM102-HER4, and SUM44 cells by puromycin resistance resulted in cell lines expressing 5R in addition to HER4. This resulted in a loss of membrane-localized HER2, as determined by immunohistochemistry (data not shown), and completely abolished heregulin-dependent HER2 tyrosine phosphorylation (Fig. 7A). Consistent with reports that expression of 5R can reduce heregulin-induced HER4 phosphorylation (4). there was a reduction in heregulin-induced HER4 tyrosine phosphorylation in SUM44 cells. The HER2 single-chain ER-tagged antibody also virtually abolished heregulin-induced HER3 phosphorylation in SUM44 cells. In SUM102-HER4 cells, expression of the 5R construct did not appreciably dampen phosphorylation of the exogenously expressed HER4 (Fig. 7B), possibly because this HER4 is expressed at high levels compared with the endogenous levels of HER4 seen in SUM44 cells, and there is essentially no detectable HER2 activation in the parental line (Fig. 5). The 5R construct did not affect the ability of EGF to induce phosphorylation of EGFR. SUM102 cells do not dem-

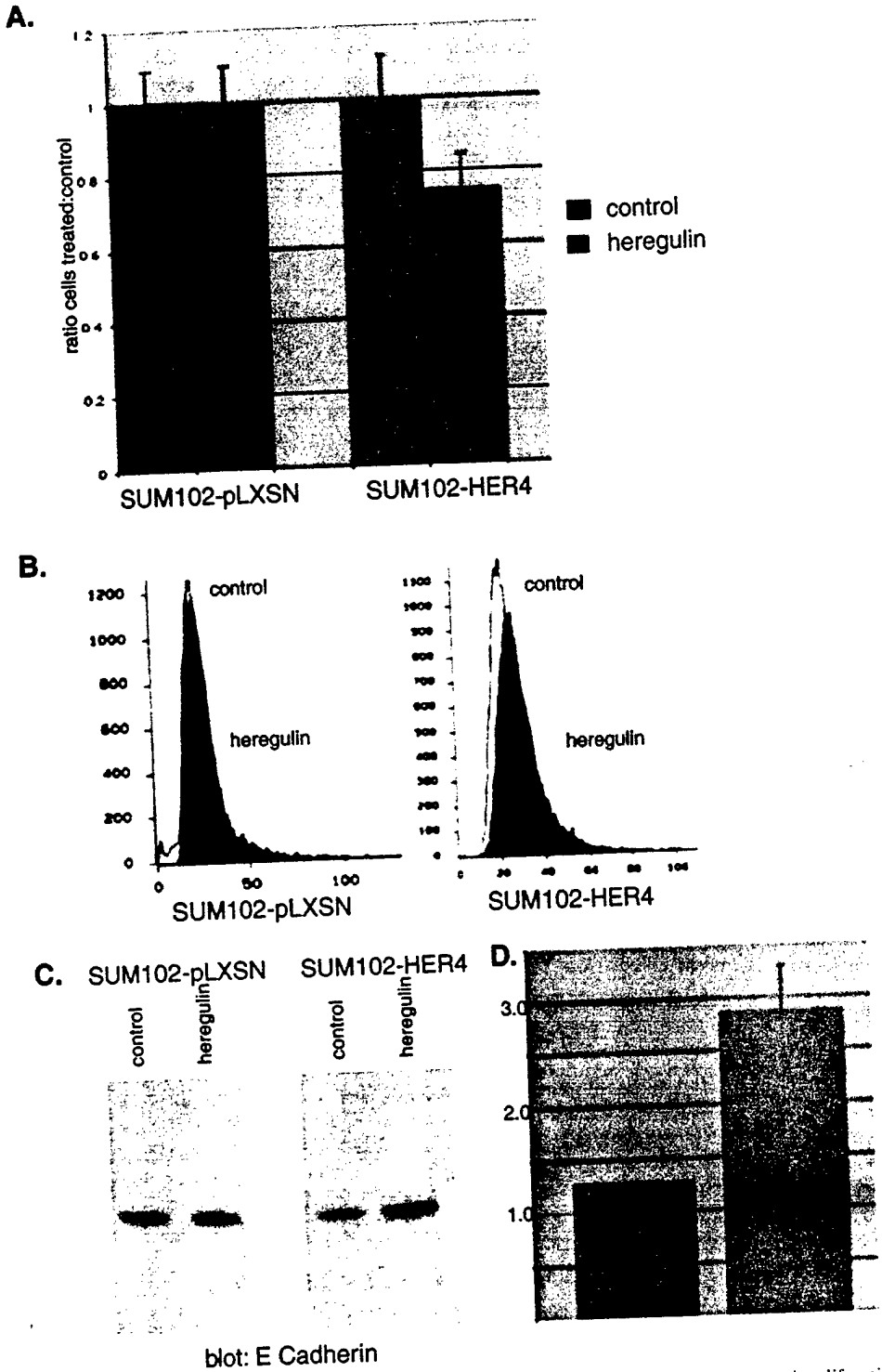


FIG. 6. SUM102 antiproliferative and differentiative response to heregulin with and without HER4. (A) Antiproliferative response. SUM102-HER4 or vector control cells were plated at a density of 5×10^5 cells per well in six-well plates and grown in the presence or absence of 10 ng of heregulin β1 per ml for three medium changes (7 days), and the number of cells was counted. The ratio of number of cells grown in the presence versus the absence of ligand is shown. Error bars represent standard deviations of at least three experiments. SUM102-HER4 cells are growth inhibited with heregulin, to an extent comparable to that of SUM44 cells. Wild-type (Fig. 1A) and vector control SUM102 cells do not have an antiproliferative response to HER4. (B) Neutral lipid production. SUM102 cells expressing vector or HER4 were treated with 10 ng of heregulin per ml for 4 to 6 days and stained with Nile red. The intensity of staining was measured by flow cytometry, and histograms of control and heregulin-treated cells were overlaid. SUM102-HER4 cells have increased neutral lipid staining when treated with heregulin, comparable to SUM44 cells, while HER4-negative control cells do not. (C and D) E cadherin expression. (C) SUM102-pLXSN vector control cells or SUM102-HER4 cells were treated with 10 ng of heregulin per ml for 4 to 6 days and lysed, and Western blotting was performed with anti-E cadherin antibody. (D) Densitometry of E cadherin expression by Western blot. Values are intensities (fold), and standard deviations of at least three experiments are shown by the error bars. SUM102-HER4 but not SUM102-pLXSN demonstrated increased E cadherin expression in response to heregulin.

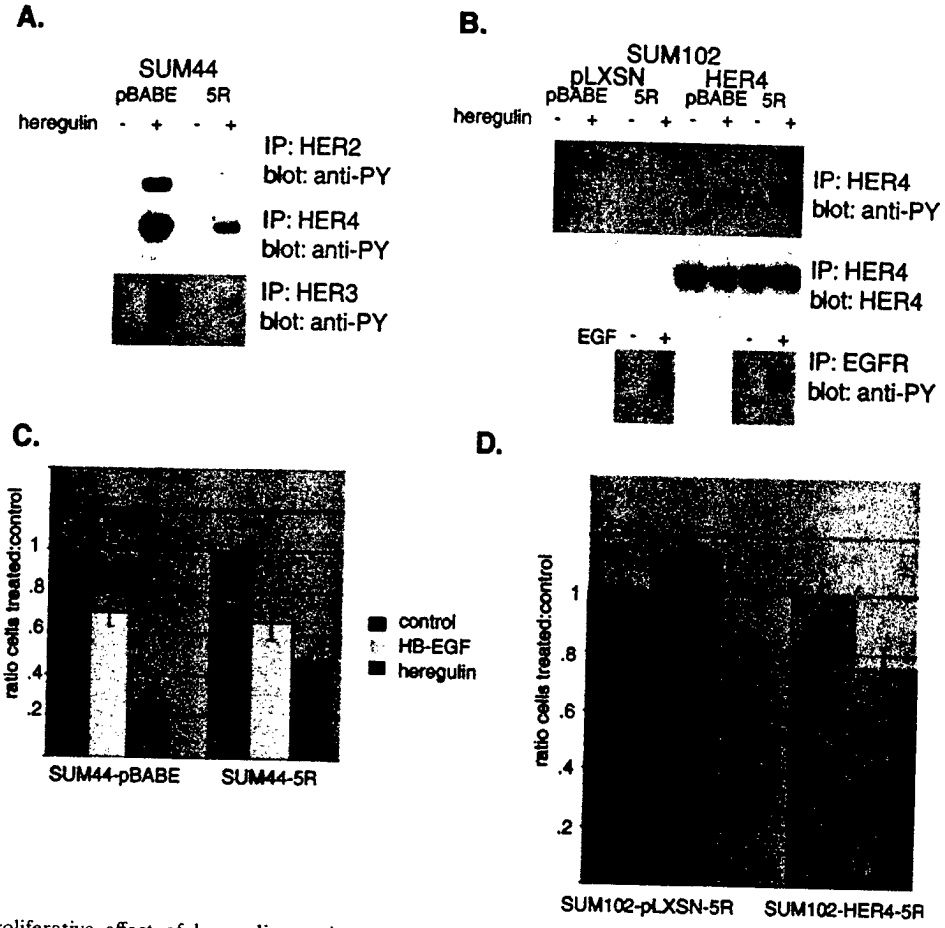


FIG. 7. The antiproliferative effect of heregulin persists even after removal of HER2 signaling. SUM44 cells and SUM102-pLXSN or SUM102-HER4 cells were infected with retrovirus containing vector alone or containing the anti-HER2 ER-tagged single-chain antibody 5R. After selection in G418, removal of HER2 from the membrane by 5R was confirmed by immunohistochemistry, demonstrating loss of HER2 membrane immunoreactivity in both (SUM44 and SUM102) 5R-containing lines (data not shown). (A) Tyrosine phosphorylation of HER2-4 in response to heregulin in SUM44 derivatives. Cells containing the 5R construct did not demonstrate heregulin-dependent HER2 tyrosine phosphorylation, as opposed to vector control cells, indicating that 5R effectively eliminates HER2 signaling in these cells. The 5R construct also inhibited heregulin-induced HER3 phosphorylation and dampened HER4 phosphorylation. IP, immunoprecipitation; anti-PY, antiphosphotyrosine. (B) Tyrosine phosphorylation of HER4 and EGFR in response to ligand stimulation in SUM102 derivatives. The 5R construct did not affect HER4 or EGFR ligand-induced phosphorylation. (C) Antiproliferative response of SUM44-5R cells. SUM44 vector control pBABE and 5R expressing cells were treated with heregulin or HB-EGF, and the proliferative response was measured as described in Materials and Methods and for Fig. 1. The absence of HER2 signaling did not alter the growth inhibitory responses of heregulin and HB-EGF. (D) Antiproliferative response to SUM102-HER4 cells. SUM102-HER4 cells or vector control cells containing 5R were treated with heregulin. Sequestration of HER2 and removal of HER2 tyrosine phosphorylation did not abolish the antiproliferative effect, and SUM102-5R cells which do not contain HER4 did not demonstrate an antiproliferative effect.

onstrate appreciable HER2 phosphorylation in response to heregulin (Fig. 5) or express HER3 (Fig. 1C).

SUM44 cells expressing the pBABE vector exhibited both HB-EGF- and heregulin-dependent antiproliferative responses. Again, heregulin was more potent. Introduction of 5R and elimination of HER2 signaling did not block either ligand-dependent antiproliferative response in SUM44 cells (Fig. 7C). In the SUM102-pLXSN cells, which do not express HER4, sequestration of HER2 did not change the lack of antiproliferative response to heregulin (Fig. 7D). Furthermore, in SUM102-HER4 cells, which had acquired an antiproliferative response to heregulin by virtue of HER4 expression, sequestration of HER2 did not abolish this response. Thus, unlike HER4, HER2 is not necessary for the antiproliferative response in cells with either endogenous (SUM44) or exogenously expressed (SUM102-HER4) HER4.

DISCUSSION

In our studies of HER4 in human breast cancer cells, we found clear antiproliferative and differentiative responses to heregulin in SUM44 cells. This response correlated with heregulin-induced HER4 tyrosine phosphorylation and was induced by another HER4 ligand, HB-EGF, which activates HER4 but not the other EGFR family members in this cell line. In addition, overexpression of kinase-dead HER4 obliterated this response. The only other cell lines that demonstrated growth suppression upon treatment with heregulin,

SUM185 and SUM225, also exhibited HER4 expression. HER4-negative cells did not show a heregulin-dependent antiproliferative response. To further confirm the involvement of HER4 in mediating an antiproliferative and differentiative response, we expressed HER4 in HER4-negative SUM102 cells. HER4-expressing SUM102 cells acquired an antiproliferative and differentiative response upon HER4 activation. Thus, HER4 can mediate antiproliferative and differentiative signals in human breast cancer cells.

Activation of HER4 and HER2 has been associated with a range of responses, including growth stimulation and suppression, as well as stimulation of expression of differentiation markers. The outcome depends upon the cell type, the complement of EGFR family members expressed, the level of HER2 expression, the ligand (and even the ligand isoform), and the presence of other growth factors or serum. Our aim was to specifically investigate the role of HER4 in the antiproliferative and/or differentiative response and to prove, to the extent possible, that HER4 activation alone was necessary and or sufficient to produce this response.

We hypothesized that if any EGFR family member was primarily responsible for the antiproliferative and differentiative response, HER4 was the likely candidate, since HER4 has been implicated in differentiation developmental responses in a number of settings. In the endometrium, HER4 expression and expression of HER4 ligands are increased during the secretory phase, suggesting a role in endometrial maturation (46). HER4 is critical for cardiac and neural development, as HER4 knockout mice are nonviable due to impaired cardiac and neural development (6, 18). In the mouse mammary gland, a carboxy-terminal deletion mutation of HER4 impairs postpartum lobuloalveolar development due to a lack of terminal differentiation (24). Consistent with a role in antiproliferation and differentiation, in human breast cancers HER4 expression is associated with low histological grade (47). This is in contrast to HER2, which is often associated with tumors with poorer prognostic features and outcome.

The complicated nature of EGFR family member interactions makes it difficult to discern the contribution of each member to the differentiation response. For example, the differentiation response to heregulin has alternately been attributed to HER2 and HER4, since heregulin can activate both receptors. We first implicated HER4 by using a ligand, HB-EGF, that does not activate HER2. To more definitely eliminate the contribution of HER2, we used single-chain antibodies that sequester HER2 in the ER. The antiproliferative response to heregulin was not abolished with HER2 loss. Our studies demonstrate that HER4 can mediate an antiproliferative signal but do not rule out a contribution from HER2 to a differentiative signal. This is consistent with the findings of others. In MCF7 cells, removal of surface HER2 affected heregulin-induced morphologic differentiation changes. However, HER2 was not required for heregulin effects on proliferation (4). Antisense HER2 expressed in AU565 cells caused cells to proliferate more slowly and abolished the antiproliferative and differentiation response to high concentrations of heregulin without affecting the proliferative response to low concentrations of heregulin (55). In AU565 cells, HER2 inhibitory antibodies induce differentiation, suggesting that removal of HER2 may enable a HER4 differentiation signal to predominate (3). HER4 agonist antibodies can induce a differentiation response, which is partially reversed by HER4 antagonist antibodies, but this is also seen with HER2 (9).

However, some studies of EGFR family member activation in 32D mouse myeloid cells support a proliferative function for HER4, since cells expressing HER4 alone or in combination with EGFR demonstrated a mitogenic response to stimulation with EGF or epiregulin (43, 49). Furthermore, downregulation of exogenously expressed HER4 by ribozymes decreased proliferation, suggesting that HER4 was mediating proliferative as opposed to antiproliferative or differentiative responses (48). However, others found that 32D cells expressing both HER2 and HER4 were growth stimulated by HB-EGF, whereas those expressing only HER4 had a growth inhibitory response, suggesting that HER4 may be involved in proliferative or antiproliferative signals, depending on presence of HER2 (52). Similarly, we have found that activation of an EGFR-HER4 chimera induced an antiproliferative response in 32D cells (data not shown).

Our studies conclusively support a role for HER4, in the absence of HER2, as a mediator of an antiproliferative and differentiative response in human breast cancer cell lines. Further investigations are under way to determine the downstream signal transduction pathways involved in HER4 signaling.

ACKNOWLEDGMENTS

This work was supported by P50CA58223 National Cancer Institute Breast Cancer SPORE, Breast Cancer Research Foundation, K08CA83753, and Department of Defense DAMD17-96-1-6015.

We thank Dominic Moore for statistical assistance, Lynn Dressler for immunohistochemical confirmation of HER2 sequestration by 5R, and Mark Day for helpful discussion.

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Appendix 2

Epidermal growth factor receptor variant (EGFRvIII) expression in a normal human breast epithelial cell line induces growth factor independence, constitutive activation of downstream signal transduction pathways, and soft agar growth.

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Running Title: EGFRvIII transformation of human mammary epithelial cells.

Key Words:

Epidermal growth factor receptor, EGFRvIII, breast cancer, oncogenes, signal transduction pathways

Abstract:

A rearranged epidermal growth factor receptor gene encodes a protein (EGFRvIII) that provides a growth advantage to gliomas *in vivo*, and in transformed cells results in ligand-independent proliferation *in vitro*. To further define the effect of EGFRvIII, we determined its capacity to transphosphorylate HER1-4 when transiently over-expressed in CHO cells and its more physiologic effect on receptor phosphorylation and cell growth when stably expressed in human mammary epithelial cells. EGFRvIII was able to functionally heterodimerize and phosphorylate HER1-4 in CHO cells, but in mammary cells only HER2 and EGFRvIII were constitutively phosphorylated. EGFRvIII expression conferred EGF and IGF-1 independence in MCF10A cells, and phosphorylation of EGFRvIII and HER2 correlated with constitutive activation of p42/p44 MAPK and Akt. Moreover, EGFRvIII expression enabled MCF10A soft agar growth and transient establishment of tumors in nude mice. Thus, EGFRvIII can induce a transformed phenotype in a normal human breast cell line; HER2 appears to be the preferred partner and may amplify EGFRvIII transforming capability.

Introduction:

The original sequencing of the viral oncogene v-erbB (the cause of avian erythroblastosis) and the epidermal growth factor receptor (EGFR) identified the EGFR as a protooncogene and v-erbB as a constitutively active tyrosine kinase with transforming capability (1). Indeed, EGFR family members are frequently over-expressed or activated by autocrine factors in a number of human tumors, including gliomas, breast, head and neck, lung, prostate, and gastrointestinal cancers. EGFR over-expression by gene amplification is seen in 40% of high grade gliomas (2), and altered products resulting from gene rearrangement have been identified. The most frequent re-arrangement, designated EGFRvIII or ΔEGFR, results in loss of exons 2-7, and functionally if not architecturally resembles v-erbB in that it has deleted sequences in the extracellular ligand-binding domain of the receptor, abrogating ligand-dependent regulation (3, 4). EGFRvIII has been reported in clinical breast cancer specimens (5, 6), and one group has recently reported that EGFRvIII, when over-expressed in the transformed MCF7 human breast cancer cell line, enhanced tumorigenicity as measured by tumor growth in nude mice and soft agar assays (7). To determine the significance and biological relevance of EGFRvIII, we studied its ability to phosphorylate other EGFR family members, activate downstream signal transducers, and the resultant phenotypic effect.

Materials and Methods

Cell lines, Tissue culture. MCF10A and SUM44 cells were grown in serum-free growth factor-defined media as previously described (8). All tissue media reagents were obtained from Sigma, except for FBS and insulin, which were obtained from Gibco,BRL.

CHO-K1 cells were grown in EMEM media containing nonessential amino acids and 10% FBS (Gibco, BRL). To create EGFRvIII-expressing lines, retroviral infection was performed as previously described (8). Briefly, full-length EGFRvIII (kind gift from Dr. D. Herlyn, Wistar Institute) was cloned into the pLXSN retroviral expression vector, and sequenced in its entirety. PA317 cells were transfected with vector alone or vector containing construct and viral supernatant collected. 1ml of viral supernatant was added with 8ug/ml polybrene to MCF10A or SUM44 cells. After a 48 hour incubation, cells were placed in medium containing G418(0.3mg/ml). G418 resistant cells were pooled and expression of EGFRvIII was confirmed by western blot.

Proliferation assays. Cells were plated into 6 well plates at a density of $5x10^4$ cells per well and grown in full growth media or without EGF or Insulin or both EGF and Insulin for 3 media changes. Cells were trypsinized and counted by hemocytometer. For growth curves, cells were counted at different time points incubated with the indicated medium constituents.

Immunoprecipitation and Immunoblot analysis. CHO-K1 cells were transiently transfected using Fugene (Roche) with EGFRvIII and EGFR, HER2, or HER4 rendered kinase inactive by site directed mutagenesis, or HER3. Cells were lysed after 24 hours and either direct lysate or post-immunoprecipitation immunoblot analysis was performed. Alternatively, G418-resistant MCF10A or SUM44 cells (as described above) were used. Immunoprecipitation and western blotting were performed as previously described (8). The antibodies used for immunoprecipitation were HER-2 (Clone 9G6.10, mouse monoclonal antibody, Neomarkers, Inc), EGFR, HER-3, and HER4 polyclonal rabbit antisera raised against the carboxyl-termina of recombinant EGFR, HER3 or HER4

expressed as a GST-fusion protein, respectively; for immunoblotting RC20 (Transduction Laboratories), erbB-2 (Ab-1, Neomarkers), EGFR, HER3, or HER4 antisera, phospho-Akt, phospho-p44/42, phospho-p38, or phospho-JNK (Cell Signaling, Beverly, MA 01915) was used.

Soft agar and tumor formation assays. 1.5ml of F-12 medium containing 0.6% agarose (Sigma) was plated into one well of six-well plate as bottom layer. After the bottom layer solidified, 20,000 cells per well were plated in a 1ml top layer containing 0.4% agarose. All samples were prepared in triplicate. Cells were incubated for 4 weeks at 37C°. Colonies were stained with MTT (5mg/ml, Sigma). Animal studies were performed by the Lineberger Comprehensive Cancer Center Human Tumor Model Core Facility in accordance with institutional animal care and use committee guidelines.
For tumor formation studies, $5x10^6$ cells (MCF10A-pLXSN or MCF10A-vIII) were injected into the flanks of nude mice, and the sites monitored weekly.

Results:

EGFRvIII-induced EGFR family member tyrosine phosphorylation.

To determine the ability of EGFRvIII to dimerize with and transphosphorylate EGFR family members, we cotransfected EGFRvIII and kinase inactive HER1-4 in CHO cells. EGFRvIII when expressed in this manner is kinase active without ligand and, as shown in Figure 1A, can tryosine phosphorylate all four family members. This establishes the potential of EGFRvIII to activate all members of the family.

To assess the interaction of EGFRvIII with the members in a more physiologic context, EGFRvIII was stably expressed in MCF10A and SUM44 cells by retroviral infection. MCF10A is a spontaneously immortalized cell line derived from normal,

human mammary epithelial cells that expresses EGFR, HER2, and HER3 at non-amplified levels. SUM44 is a human breast cancer cell line that expresses HER2-HER4. Immunoprecipitation and blotting were performed using vector control or EGFRvIII expressing cells, in the presence or absence of ligand stimulation (Figure 1B and C). Constitutive tyrosine phosphorylation of EGFRvIII was observed in EGFRvIII-expressing cells (MCF10A-vIII and SUM44-vIII), but was not further enhanced by stimulation with heregulin or EGF. In contrast, EGFR was not phosphorylated in the EGFRvIII-expressing cells or vector control cells until ligand was added. Thus, in these cells EGFRvIII expression does not induce ligand-independent EGFR tyrosine phosphorylation.

In addition to constitutive EGFRvIII phosphorylation, low-level HER2 tyrosine phosphorylation was seen in EGFRvIII-expressing but not vector control cells (Figure 1B and C). With ligand stimulation by either heregulin or EGF, there was further induction of HER2 tyrosine phosphorylation in EGFRvIII-expressing cells, similar to vector control cells. Thus, EGFRvIII expression was associated with constitutive HER2 tyrosine phosphorylation, but did not abrogate further induction by ligand stimulation. In contrast to HER2, and similar to EGFR, HER3 was not constitutively activated in EGFRvIII-expressing cells, but was activated in response to heregulin stimulation. To determine whether EGFRvIII induced ligand-independent phosphorylation of HER4, we stably expressed EGFRvIII in SUM44 cells, a breast cancer cell line that expresses HER2, HER3, and HER4. As in the MCF10A cells, expression of EGFRvIII induced minimal but definite ligand-independent HER2 phosphorylation. Neither HER3 nor HER4 were constitutively phosphorylated (Figure 1C).

These results indicate that while overexpressed EGFRvIII can transphosphorylate HER1-4 when overexpressed, in a mammary epithelial cell line that expresses normal levels of HER1-3, EGFRvIII only induces constitutive activation of HER2.

EGFRvIII enables growth factor independent proliferation.

Since expression of EGFRvIII induced constitutive activation of EGFRvIII and HER2, we wished to see whether expression induced phenotypic changes in MCF10A cells. Growth factor independence is a hallmark of pathologic proliferation of human mammary epithelial cells, and MCF10A cells are strictly dependent on exogenous EGF and insulin for growth in serum-free conditions. MCF10A cells were grown in fully supplemented, serum-free, growth factor defined medium, or in serum free medium with EGF or insulin deleted. MCF10A vector control cells displayed limited growth in the absence of insulin, and essentially no proliferation in the absence of EGF or absence of both growth factors (Figure 2A). In contrast, MCF10A-vIII cells proliferated in the absence of EGF. They were also partially resistant to insulin starvation. Interestingly, when both growth factors were restricted, MCF10A-vIII proliferation was sharply reduced.

To further characterize the growth factor-independent proliferation of MCF10A cells, growth curves were generated by counting cells at various timepoints (Figure 2B). Expression of EGFRvIII did not confer a proliferative advantage in fully-supplemented medium; the proliferative rate of MCF10A-vIII was less than vector control cells. However, under conditions of growth factor deprivation (EGF starvation), MCF10A-vIII were able to proliferate at virtually the same rate with or without EGF, and thus grew

substantially better than vector control cells in the absence of EGF, confirming the EGF-independent phenotype conferred by expression of EGFRvIII in MCF10A cells.

Downstream signal transduction pathways induced by EGFRvIII expression in mammary epithelial cells.

EGFR family member activation may elicit a proliferative response via several downstream signal transduction pathways, including those of the MAPK and PI3K family pathways. We examined the effect of ligand activation or EGFRvIII expression on the activity of ERK, JNK, p38, and PI3K cascades. Akt, a downstream mediator of PI3K signals, was not constitutively phosphorylated in growth-factor starved MCF10A vector control cells (Figure 3). In contrast, MCF10A-vIII demonstrated constitutive Akt phosphorylation in the absence of EGF and insulin. In both the control MCF10A and MCF10AvIII cells, heregulin induced further Akt tyrosine phosphorylation above the constitutive phosphorylation. In vector control cells, EGF induced Akt phosphorylation, but in EGFRvIII-expressing cells the induction of Akt phosphorylation over the baseline constitutive activation was minimal.

Similarly, p44/42 MAPK was constitutively phosphorylated under growth factor-deprived conditions, whereas control cells demonstrated no baseline p44/42 MAPK phosphorylation. As with Akt, ligand stimulation with either heregulin or EGF induced p44/42 MAPK phosphorylation in vector control cells. However, while heregulin stimulation induced further tyrosine phosphorylation over constitutive activation of p44/42 MAPK in MCF10AvIII, EGF stimulation did not induce substantial increase in phosphorylation over baseline.

In contrast to p44/42 MAPK, there was no difference in p38MAPK phosphorylation, either in growth factor-deprived or ligand stimulated conditions. There was basal p38 MAPK phosphorylation in both MCF10A vector control and EGFRvIII-expressing cells, uninduced by ligand stimulation. However, there were differences in JNK phosphorylation between vector control cells and EGFRvIII-expressing cells. In vector control cells, JNK was not constitutively phosphorylated, but phosphorylation was induced by both EGF and heregulin stimulation. In contrast, expression of EGFRvIII abolished the ligand-dependent phosphorylation of JNK.

EGFRvIII expression induces a transformed phenotype.

To evaluate whether expression of EGFRvIII in MCF10A cells conferred features of malignant transformation, we investigated whether MCF10A-vIII cells would grow under anchorage-independent conditions. Vector control cells or MCF10AvIII cells were grown in soft agar. At two weeks, there were no obvious colonies in either EGFRvIII-expressing or control cells. However, after four weeks, numerous colonies of MCF10AvIII cells developed, while no colonies developed of vector control cells at 4 weeks or by eight weeks (Figure 4A). Thus, EGFRvIII expression confers anchorage-independent growth in MCF10A cells.

To determine whether expression of EGFRvIII enabled MCF10A cells to form tumors in nude mice, mice were injected with vector control MCF10A cells or MCF10A-vIII cells. Neither vector control nor EGFRvIII cell injections formed durable tumors in nude mice. However, unlike mice injected with MCF10A-pLXSN control cells, mice injected with MCF10A-vIII cells formed transient tumor masses at the injection site

within one week (Figure 4B). This transient growth and rapid regression was reproducibly observed.

Discussion:

To determine the significance and biological relevance of EGFRvIII, we studied its ability to phosphorylate other EGFR family members, and the resultant phenotypic effect. The EGFRvIII-induced phenotypes of growth factor-independent proliferation, enhanced tumorigenicity, and anchorage-independent growth have been attributed to low-level, constitutive phosphorylation of EGFRvIII, thought to result from a conformational change that permits ligand-independent homodimerization (9-11). Consistent with other reports, we found constitutive phosphorylation of EGFRvIII in MCF10AvIII cells, which was not further induced by EGF. However, we also found ligand-independent HER2 phosphorylation in EGFRvIII-expressing MCF10A cells, as has been seen in MCF7 cells expressing EGFRvIII (7). Together, these studies suggest that EGFRvIII:HER2 heterodimers may form when EGFRvIII is expressed at modest levels in human breast epithelial cells.

Interestingly, when expressed in MCF10A cells, EGFRvIII did not induce constitutive tyrosine phosphorylation of endogenous EGFR. Likewise, wt EGFR is not phosphorylated when co-expressed with EGFRvIII in glioma cells (12). In contrast, overexpression of EGFRvIII to high levels was able to induce phosphorylation of kinase inactive EGFR in CHO cells. Thus, the ability of EGFRvIII to heterodimerize with and phosphorylate wtEGFR may depend on the level of expression, or presence of other more favored heterodimerization partners, such as HER2. Similarly, EGFRvIII expression did not induce phosphorylation of endogenous HER3 or HER4, although phosphorylation

could be demonstrated when kinase inactive forms of these receptors were expressed at high levels in CHO cells. Therefore, at least in MCF10A cells, activation of EGFRvIII and/or HER2, but not EGFR, HER3, or HER4 correlated with acquisition of phenotypic changes.

Despite constitutive activation of EGFRvIII and HER2, we did not see a proliferative advantage in MCF10AvIII cells under growth factor-complete conditions. In fact, these EGFRvIII-expressing cells consistently grew more slowly than vector control cells. In contrast, under EGF-deprived conditions MCF10AvIII cells demonstrated a clear proliferative advantage. EGFRvIII expression has been shown to confer a ligand-independent proliferative advantage in malignant glioma cells and human breast cancer, and EGFRvIII expression obviates the need for IL-3 in IL-3-dependent 32D cells (7) (9, 12). Likewise, EGFRvIII expression has been shown to increase proliferation and reduce apoptosis of glioma cells grown in nude mice, and to mediate resistance to chemotherapy (9, 13).

We demonstrate that EGFRvIII expression in MCF10A cells induces ligand-independent constitutive activation of Akt, implying constitutive activation of PI3K. EGFRvIII-induced PI3K activation has also been demonstrated in NIH-3T3 cells, and is thought to be responsible for the serum- and anchorage-independent growth in EGFRvIII-expressing NIH3T3 cells (14). In these cells, PI3K activation is required for constitutive JNK activation as determined by inhibition of constitutive JNK activation when EGFRvIII-expressing cells are treated with LY294002 (15). Despite constitutive Akt phosphorylation, we did not see constitutive JNK activation. If anything, we see a decrease in heregulin-induced JNK activation.

EGFRvIII enabled MCF10A cells to form palpable growth in nude mice compared to vector-expressing MCF10A cells. However, these were not durable. On the other hand, EGFRvIII did promote growth in soft agar. Thus, EGFRvIII enables anchorage-independent survival and growth, but not full, durable tumorigenicity in normal mammary epithelial cells. The enhanced tumorigenicity of EGFRvIII-expressing gliomas has been attributed to activation of the ras-SHC-grb2 pathway, since ras-neutralizing antibodies obliterate the proliferative signal and SHC phosphorylation is dependent on the kinase activity of EGFRvIII (16). Our finding of constitutive p44/42 MAPK in EGFRvIII-expressing MCF10A cells is consistent with these results. In contrast, in NIH3T3 cells EGFRvIII expression appears to downregulate the ras-MAPK pathway, presumably via upregulation of phosphatase activity (10, 15) (17). One explanation for these different results may be that EGFRvIII expression in the presence of other EGFR family members may elicit different downstream signal transduction pathways than when expressed alone. For instance, EGFRvIII:HER2 heterodimerization (Figure 1B) may be responsible for the constitutive p44/42 phosphorylation seen in EGFRvIII-expressing MCF10A cells. Alternatively, cell-type specific differences in phosphatase activity may account for the disparate observations, such as the suppression of JNK activation in EGFRvIII expressing MCF10A cells.

We have shown that, in addition to enhancing malignant features of tumor cells, EGFRvIII can function to transform MCF10A non-malignant human mammary epithelial cells. Since EGFRvIII expression resulted in constitutive activation of both itself and HER2, the downstream signal transduction pathways and resultant phenotype may be a result of either HER2 activation or EGFRvIII activation, or both.

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Legends:

Figure 1. **EGFRvIII-induced HER1-4 tyrosine phosphorylation.** A) CHO-K1 cells were co-transfected with 4ug vector DNA, 2 ug vector plus 2ug EGFRvIII DNA , or 2ug EGFRvIII plus 2ug of EGFR, HER2, or HER4 constructs made kinase inactive by mutation of the ATP binding lysine (kdEGFR, kdHER2, or kdHER4), or the normally kinase inactive HER3. Phosphotyrosine and receptor antibody immunoblots are shown that demonstrate EGFRvIII autophosphorylation and its tyrosine phosphorylation of kinase inactive HER1-4. For EGFR lysate immunoblots were used because our carboxy-terminal antisera does not distinguish EGFR and EGFRvIII. For HER2-4 the dual transfected cells were homogenized, split into two aliquots and immunoprecipitated with EGFR and the respective HER2-4 antibody prior to gel electophoresis and immunoblotting. EGFRvIII induced phosphorylation of itself and each of the EGFR family members. MCF10A cells (B) or SUM44 cells (C) stably expressing EGFRvIII or vector control were starved for 24 hours (control) or stimulated with 100ng/ml heregulin B1 or 10ng/ml EGF for 15', immunoprecipitated with either anti-EGFR, anti-HER2, anti-HER3, or anti-HER4 and blotted with anti-phosphotyrosine. EGFRvIII was constitutively phosphorylated, as was HER2.

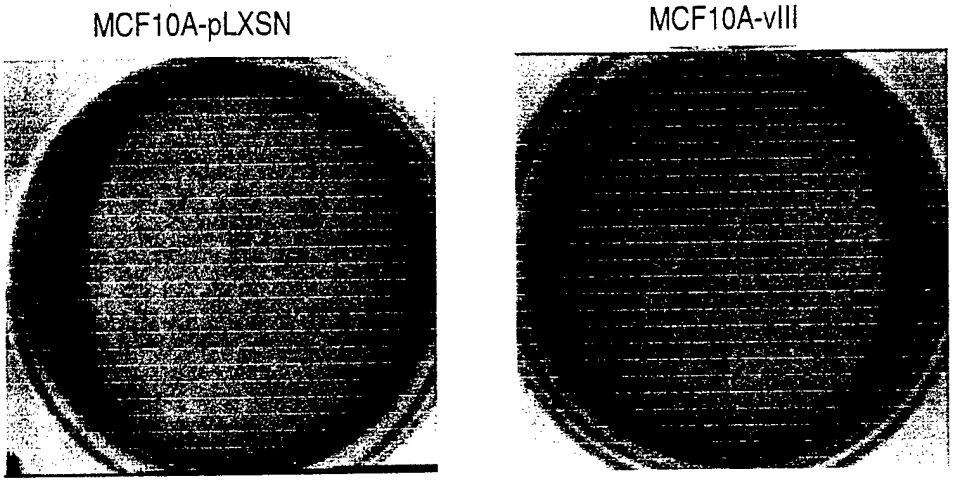
Figure 2. **EGFRvIII-induced growth factor independent proliferation.** A) MCF10A-pLXSN or MCF10A-vIII cells were grown in growth factor complete, EGF-, IGF-, or EGF- and IGF-deficient medium for 7 days (3 media changes). Cells were counted and ratio of cells in growth factor-deficient vs. complete medium is shown. Cells were grown

in complete (B) or EGF-deficient medium (C) and counted at the times shown. Although EGFRvIII-expressing cells grew more slowly in complete media than vector control cells, EGFRvIII enabled growth factor-independent proliferation.

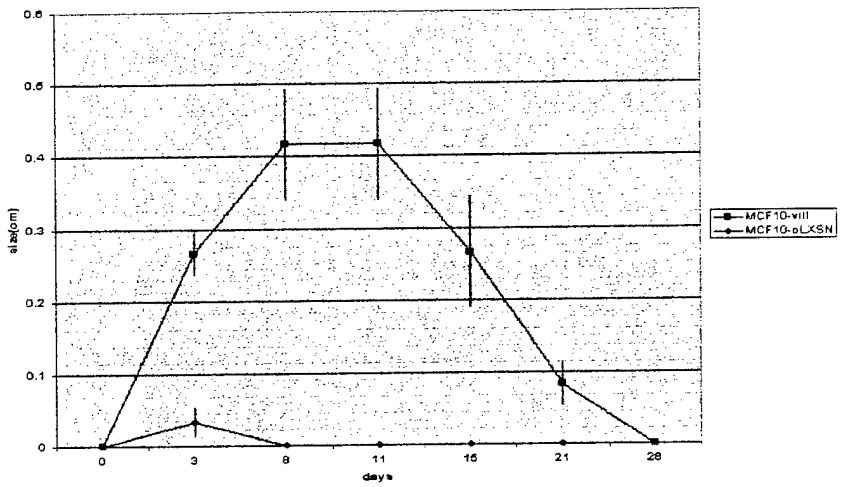
Figure 3. **Downstream signal transduction pathways induced by EGFRvIII expression.** MCF10A-vIII expressing or vector control cells were withdrawn from EGF and insulin for 24 hours and unstimulated (control) or stimulated with heregulin or EGF for 15 minutes, lysed, electrophoresed, and blotted with antibodies against the phosphorylated forms of Akt, p44/42 MAPK, p38 MAPK or JNK. EGFRvIII expressing, but not control cells demonstrated constitutive phosphorylation of Akt and p44/42 MAPK. EGFRvIII-expressing cells demonstrated reduced JNK phosphorylation in response to ligand stimulation.

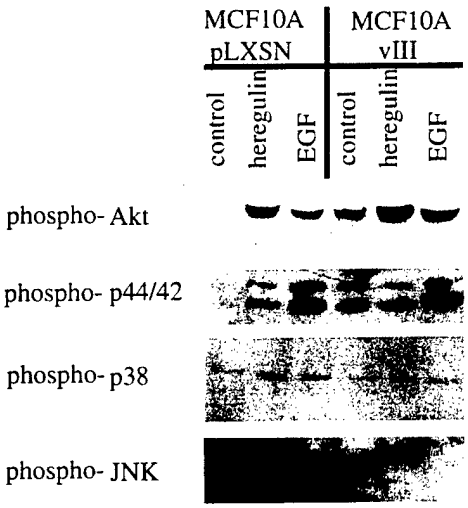
Figure 4. **EGFRvIII-induced soft agar growth and transient tumor formation in nude mice.** A) MCF10A-pLXSN or MCF10A-vIII cells were grown in complete medium in soft agar. MCF10A-vIII cells formed multiple colonies under anchorage-independent conditions, while MCF10A-pLXSN were unable to form colonies. B) MCF10A-vIII cells were injected into nude mice. Tumor volume as determined by 3-dimensional measurements vs. time since injection is shown as an average of all tumors. MCF10A-vIII cells formed small, transient tumors.

A.

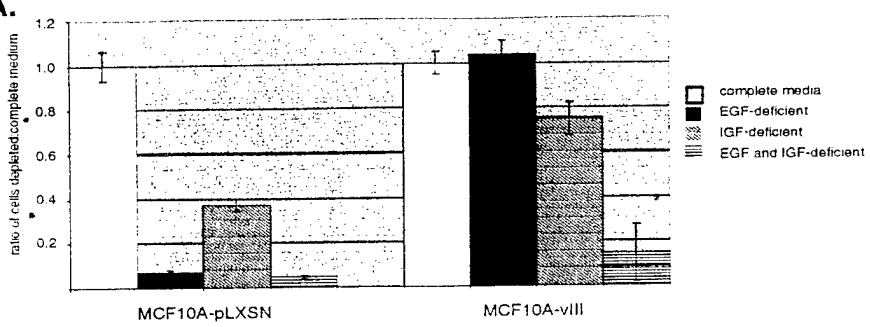

MCF10A-pLXSN
MCF10A-vIII

B.

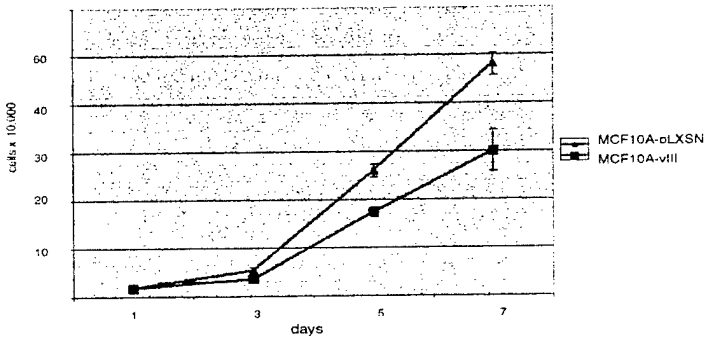

0.6
0.5
0.4
0.3
0.2
0.1
0
0
3
8
11
15
21
28
days
MCF10-vIII
MCF10-pLXSN


MCF10A pLXSN
MCF10A vIII
control
heregulin
EGF
control
heregulin
EGF
phospho- Akt
phospho- p44/42
phospho- p38
phospho- JNK

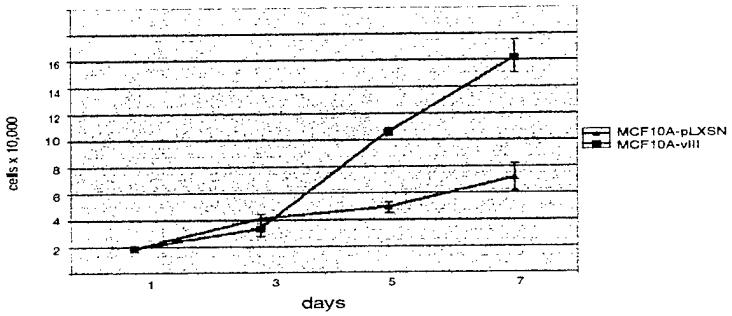
A.


ratio of cells depleted:complete medium
1.2
1.0
0.8
0.6
0.4
0.2
MCF10A-pLXSN
MCF10A-vIII
complete media
EGF-deficient
IGF-deficient
EGF and IGF-deficient

B.

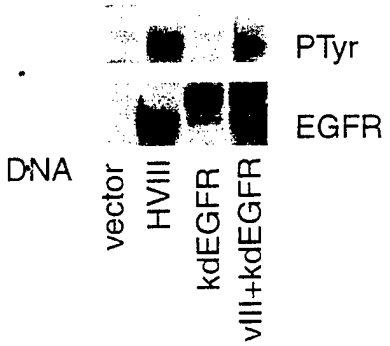

cells x 10,000
60
40
30
20
10
1
3
5
7
days
MCF10A-pLXSN
MCF10A-vIII

C.

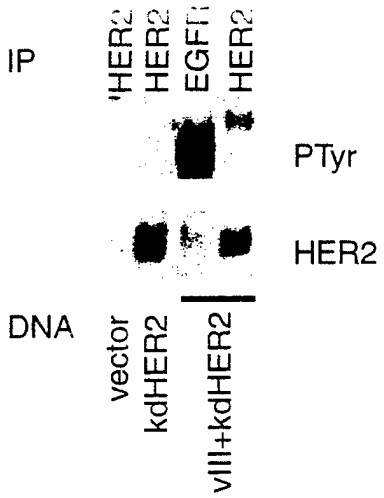

cells x 10,000
16
14
12
10
8
6
4
2
1
3
5
7
days
MCF10A-pLXSN
MCF10A-vIII

A.

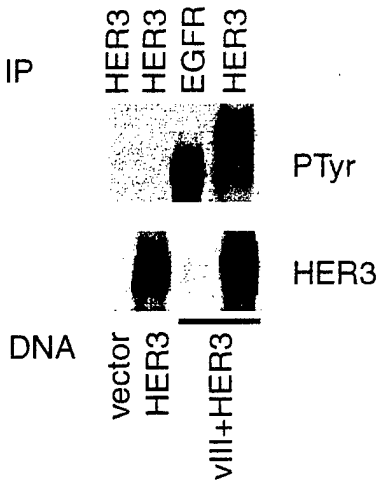
1. Lysate Immunoblot, EGFR



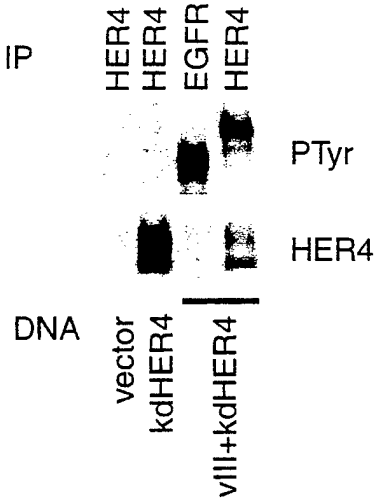
2. Immunoprecipitation, HER2



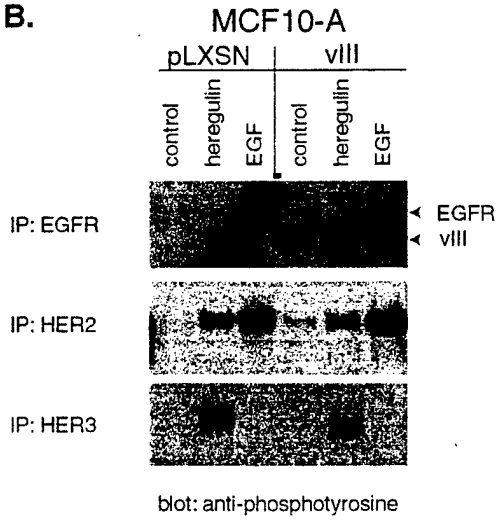
3. Immunoprecipitation, HER3



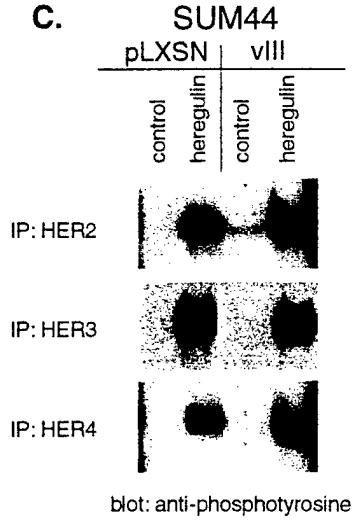
4. Immunoprecipitation, HER4



B.



C.



Appendix 3

Background and Objectives

It is well established that up to 50% of Glioblastomas amplify the EGFR gene with resultant overexpression of the receptor protein[1-3]. In these tumors, the amplified EGFR gene frequently reveals various deletions between exons 2 and 7 that result in a single splice variant encoding EGFRvIII (vIII)[4]. Splicing base-pair 274 (exon1) to bp 1076 (exon8) truncates the first two segments of the receptor ligand-binding domain (aa 6-276) and introduces a novel glycine at the fusion point. This 145 Kda truncated receptor does not bind ligand and is constitutively activated. Overexpression of vIII in cell lines results in enhanced growth, transformed phenotype and tumorigenicity in nude mice[5-7]. Interest generated by these biologic attributes coupled with the novel glycine at the fusion point resulted in development of polyclonal and monoclonal antibodies that bind vIII.

Using an affinity purified rabbit polyclonal antibody, it has been published that 78 % of breast tumors express vIII by western blot and 27% by immunohistochemistry [8, 9]. The same reagent revealed that as prostate carcinomas increase in grade, immunodetectable vIII protein levels increase [10].

Author	Samples	Method	% Positive
Moscatello	27 Breast Ca	Polyclonal Ab (Affinity Purified) Western 100ug (100 Kda band)	78%
Wikstrand C J	11 Breast Ca	Monoclonal Immunohistochemistry	27%
Olapade EO	31 Prostate Ca 12 Metastasis	Polyclonal Ab (Affinity Purified) 1-Immunohistochemistry 2-Western 250ug (148 Kda band)	AICaP > ADCaP (68%) cytoplasmic, perinuclear staining

Obviously, these findings could have important treatment implications for patients with these malignancies. We were also intrigued by the lack of definitive evidence for EGFR gene amplification or protein overexpression in either breast or prostate tumors.

Availability of exquisitely sensitive and specific real-time fluorescent quantitative PCR presents the opportunity to study EGFR and vIII mRNA expression levels in both breast and prostate carcinomas. We also wished to correlate mRNA levels with protein expression data.

MATERIALS AND METHODS

TISSUE SPECIMENS

- Tumor samples and matched normal tissue controls were procured in the operating room and were snap-frozen in liquid nitrogen. Tissue immediately adjacent to the frozen tumor was embedded in paraffin, sectioned, and hemotoxylin and eosin stained for quality control.
- **Breast**: **27** invasive breast carcinomas and matched normal controls.
- **Prostate: 13** androgen independent prostate carcinomas, **19** androgen-dependent prostate carcinomas and **12** matched normal controls.

RNA ISOLATION AND QUANTITATION

- Specimens were mechanically homogenized and RNA was extracted using a Guanidinium Isothiocyanate based protocol.
- Contaminating Genomic DNA was removed by DNAse digestion (2U/µl, Rnase free) for 30 min. at 37 °C. DNAse enzyme was than removed by purification over Rneasy columns (Qiagen).
- RNA concentration and purity was initially determined by absorbance at 260/280. After dilution to 10ng/µl, RNA was again quantified using Ribogreen fluorescence quantitation.

- EGFR was measured using 10ng of total RNA per reaction. Given the presumed lower concentration, 40ng of total RNA were used for vIII assays. Tumors and matched normal controls were assayed in triplicate using the same reagent master mix.
- 3 fold dilutions of gene specific, synthetic RNA (sRNA) were used as positive control and absolute standard.

RT-PCR REACTION MIX

Component	Final Concentration
10X Taqman buffer	1X
25 mM $MgCl_2$	5.5 mM
10 mM deoxyATP	300 μM
10 mM deoxyGTP	300 μM
10 mM deoxyCTP	300 μM
20 mM deoxyUTP	300 μM
10 μM forward primer	300 nM
10 μM reverse primer	300 nM
20 μM probe	200 nM
AmpliTaq Gold DNA Polymerase (5U/μl	0.025 U/μl
Multiscribe Reverse Transcriptase (50U/μl)	0.25 U/μl
Rnase Inhibitor	0.4 U/μl

IMMUNOPRECIPITATION

- Tissue samples were lysed in NLB (20 mM Tris pH7.5, 1%TritonX-100, 500 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3V0_4, 10% Glycerol, 10 mg/ml PMSF, 10 mg/ml Leupeptin, 4 µl/ml Aprotinin).
- After sonication for 5 seconds, nuclei and insoluble material were removed by centrifugation at 13,000 x g for 15 minutes at 4°C.
- Using 500 µg of lysate, receptor proteins were precipitated with 25µl of Protein A Agarose + 5µL of Ab22 (polyclonal rabbit antisera raised against recombinant glutathione S-transferase fusion protein containing the C-terminal 100 amino acids of EGFR) for 3 h at 4°C.
- Immune complexes were washed three times with lysis buffer then denatured in sodium dodecyl sulfate sample buffer.

PAGE AND IMMUNODETECTION

- Protein samples were separated on a SDS-8% or 12% polyacrylamide gel then electrophoretically transferred to a PVDF membrane.
- After blocking for 1 hour with 3% cold fish gelatin in Tris buffered saline-0.1% Tween-20, membranes were incubated with 1:1000 dilution of AB22 (anti-EGFR) or DH8.3 (monoclonal anti vIII, AbCam).
- Membranes were washed then incubated with anti-rabbit (1:10000) or anti-mouse (1:5000) secondary antibody.
- Detection was performed by ECL.

EGFR, VIII sRNA Constructs

Gene	Vector	Cloning Sites	Linearization Site	MW
EGFR	pcDNA3	XbaI	ScaI	1257116
VIII	pcDNA3	Kpn	XhoI, XpaI	

EGFR and vIII Primer- Probe Sets

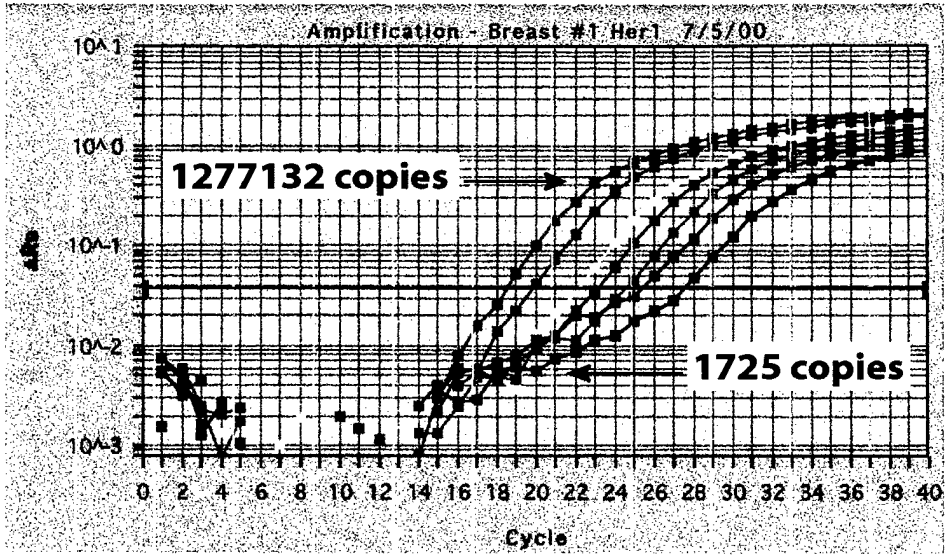
Gene	Forward primer	Reverse primer	Probe*
EGFR	tgcagaatcctgtctatcacaatca	agggctgtcgaatgtgctgtt	accccgagtatctcaacactgtccagc
VIII	tggaggaaaagaaaggtaattatgtg	ggcccttcgcacttcttaca	tgacagatcacggctcgtgcgtc

5'-Fam and 3'-TAMRA

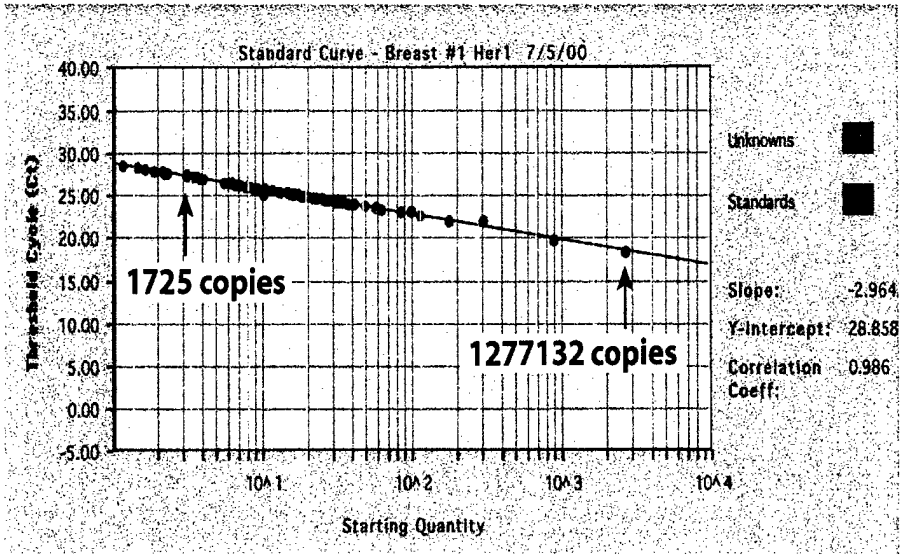
Thermal Cycling Parameters

Step:	Reverse Transcription	AmpliTaq Gold Activation	PCR (40 cycles) Denature	PCR (40 cycles) Anneal/Extend
Time:	30 min.	10 min.	15 sec.	1 min.
Temp:	48 °C	95 °C	95 °C	60 °C

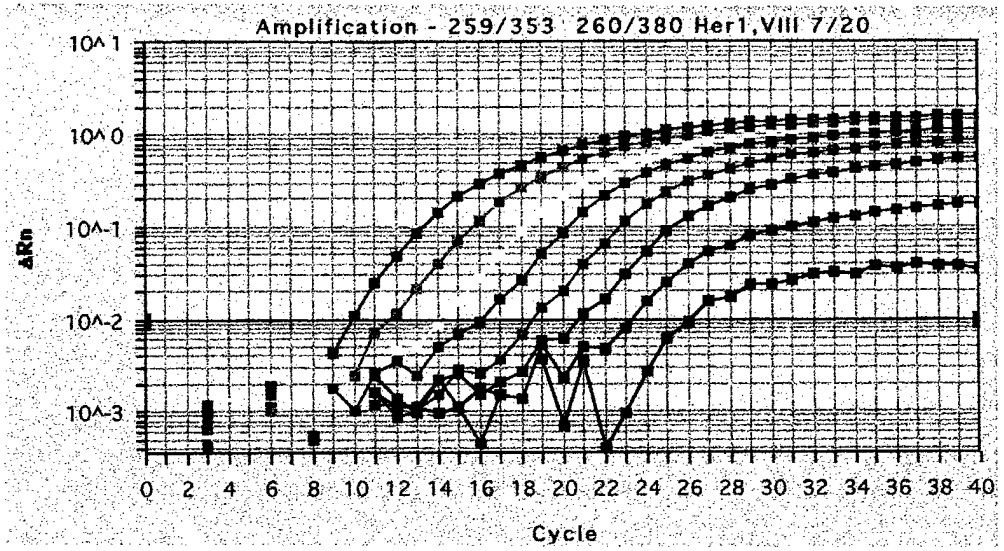
HER1 (EGF receptor) absolute standards amplification plots.



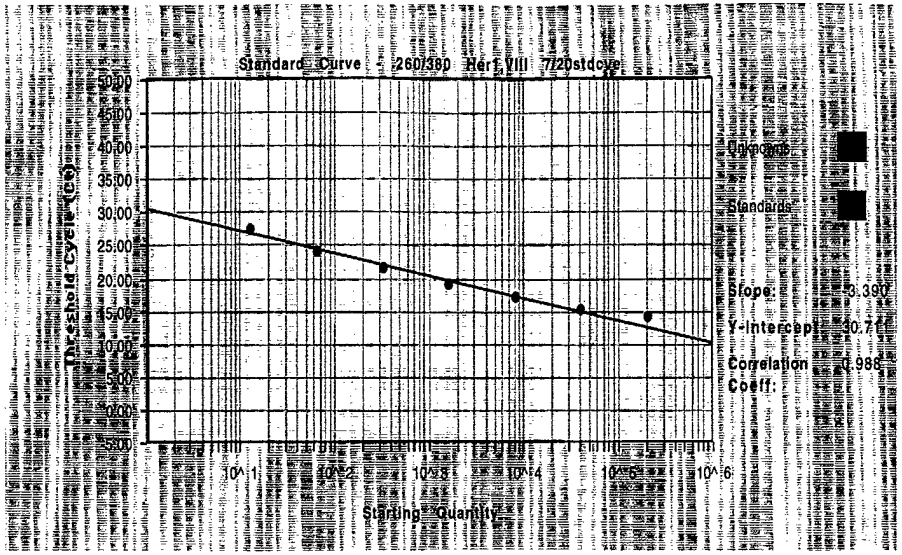
HER1 (EGF receptor) absolute standards (black dots) and tumor samples (red dots).



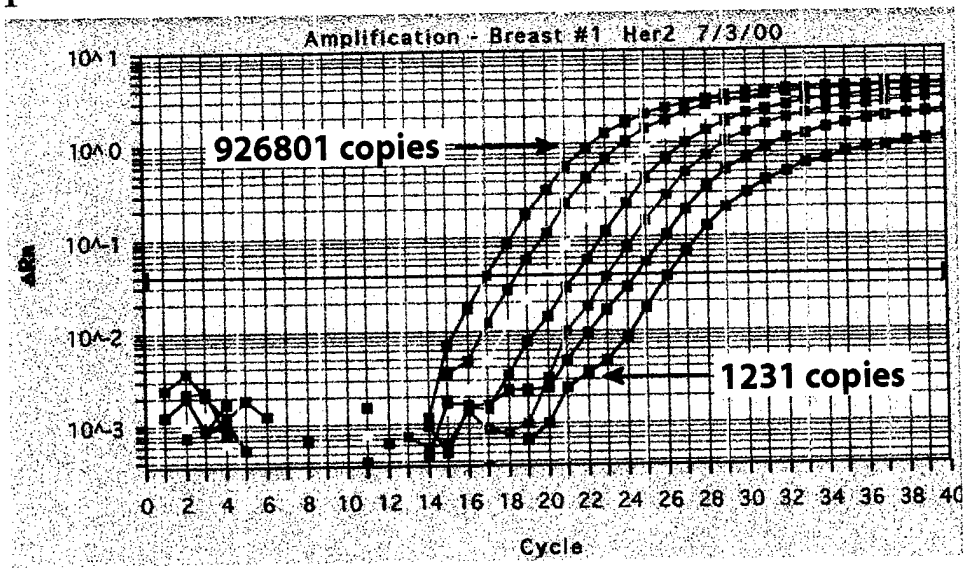
EGFR-vIII absolute standards amplification plots.



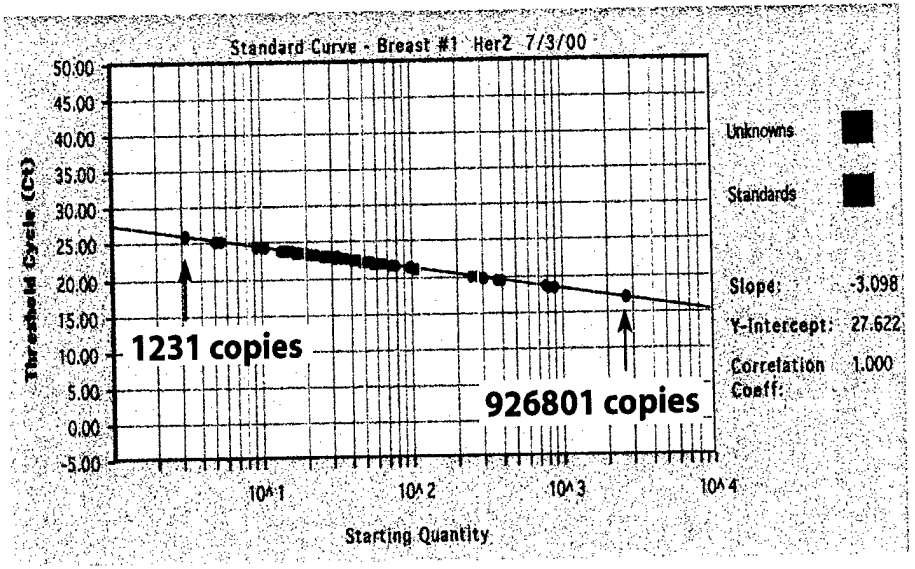
EGFR-vIII absolute standards.



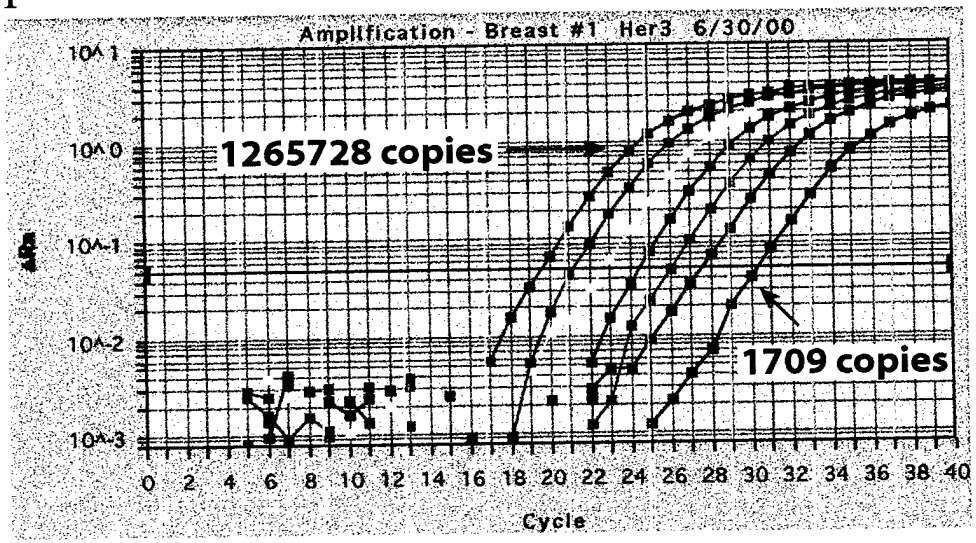
HER2 absolute standards amplification plots.



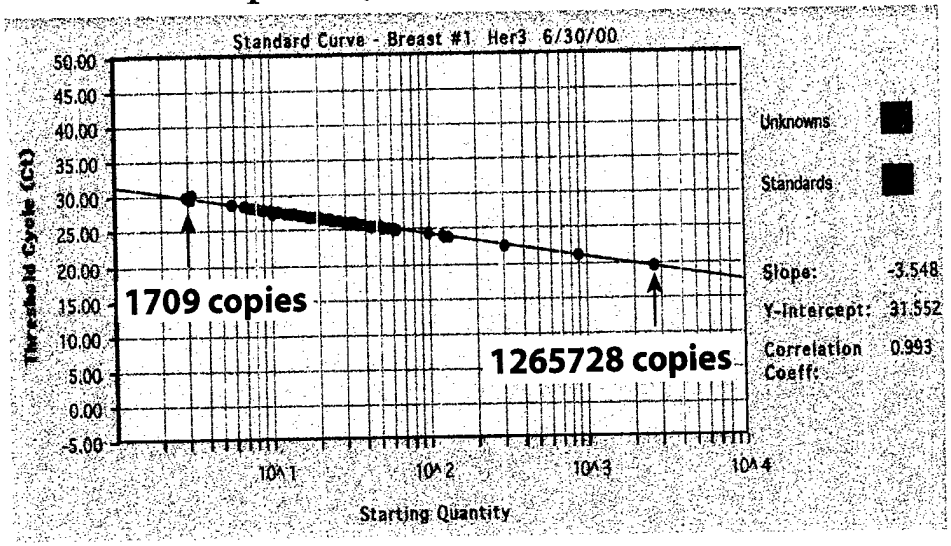
HER2 absolute standards (black dots) and tumor samples (red dots).



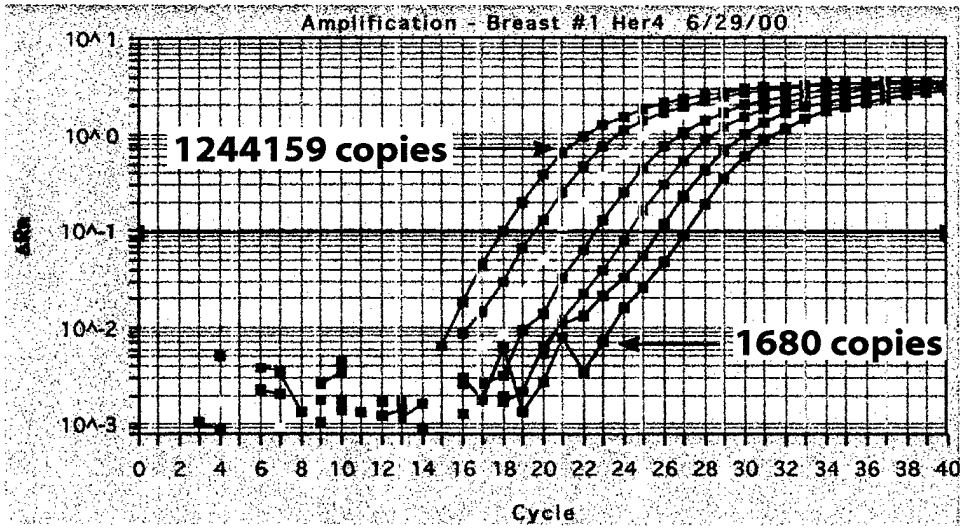
HER3 absolute standards amplification plots.



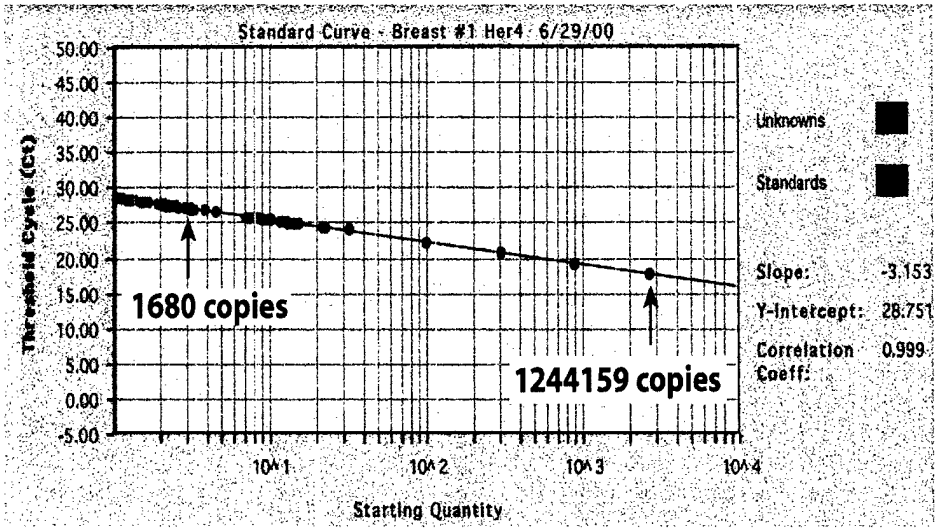
HER3 absolute standards (black dots) and tumor samples (red dots).



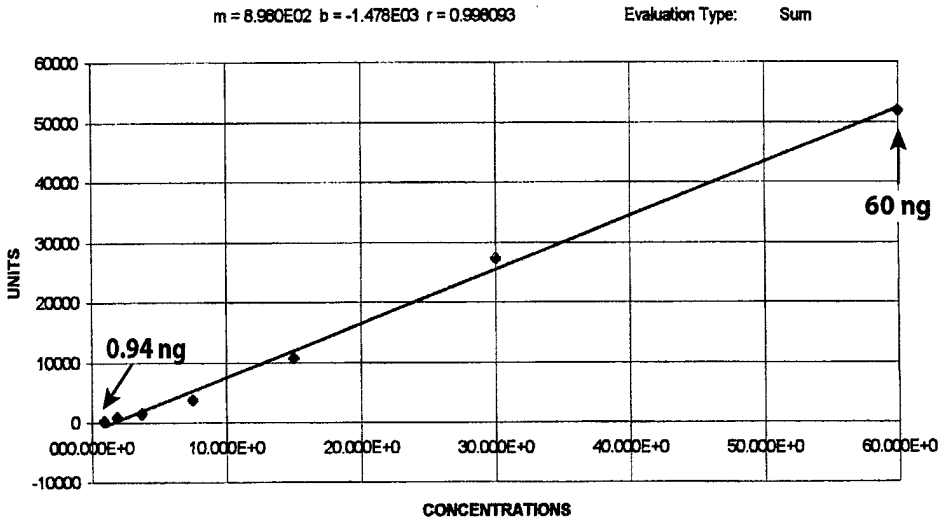
HER4 absolute standards amplification plots.



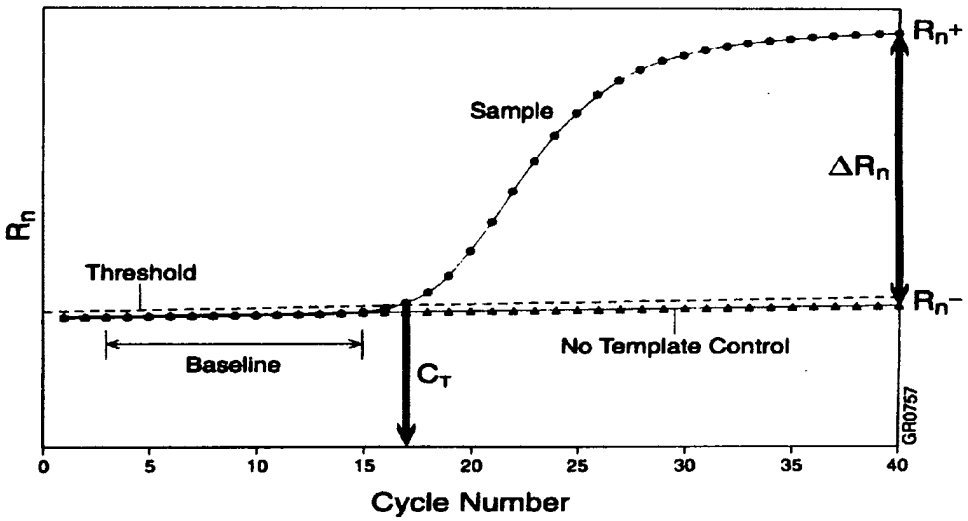
HER4 absolute standards (black dots) and tumor samples (red dots).

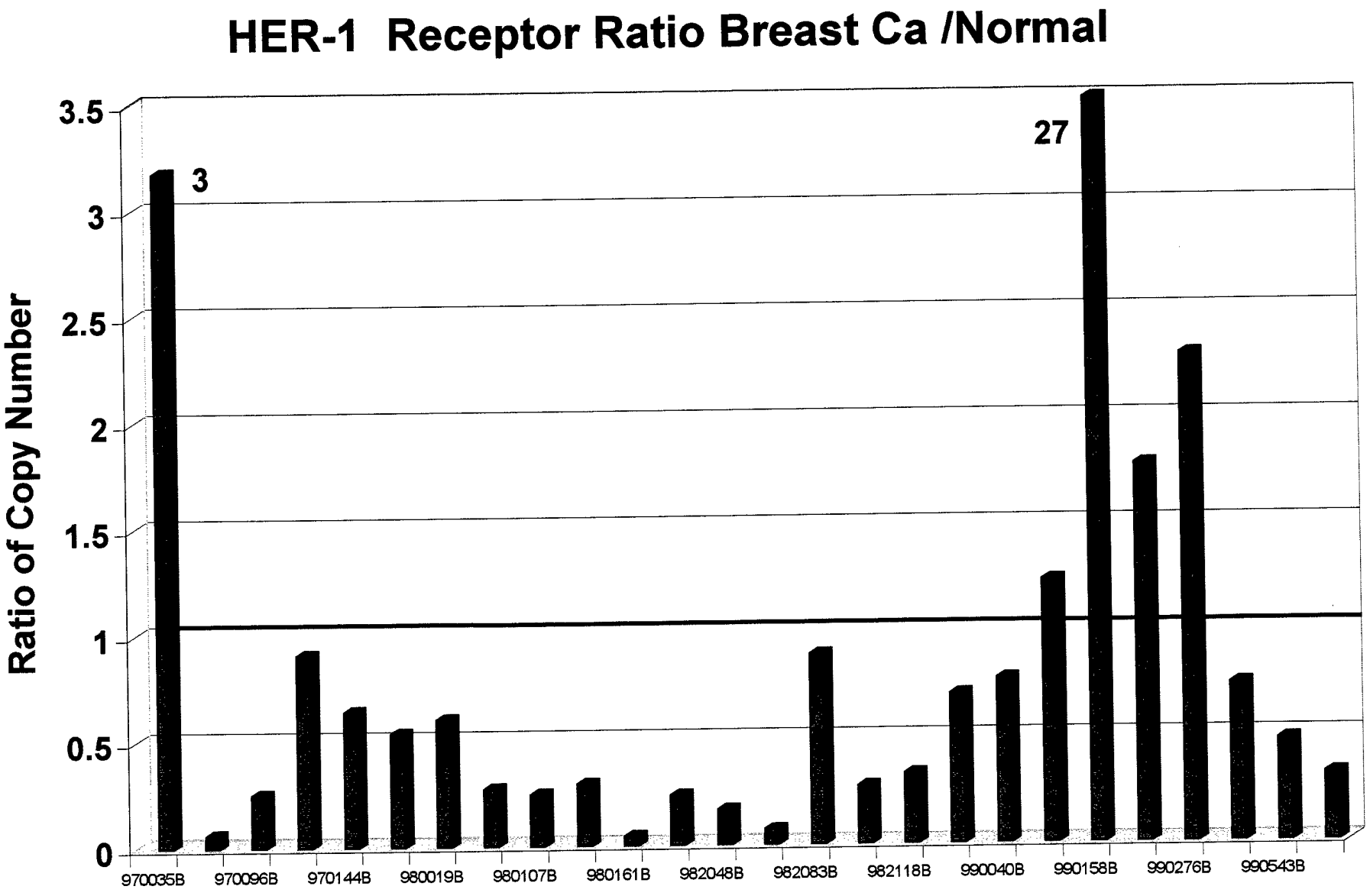


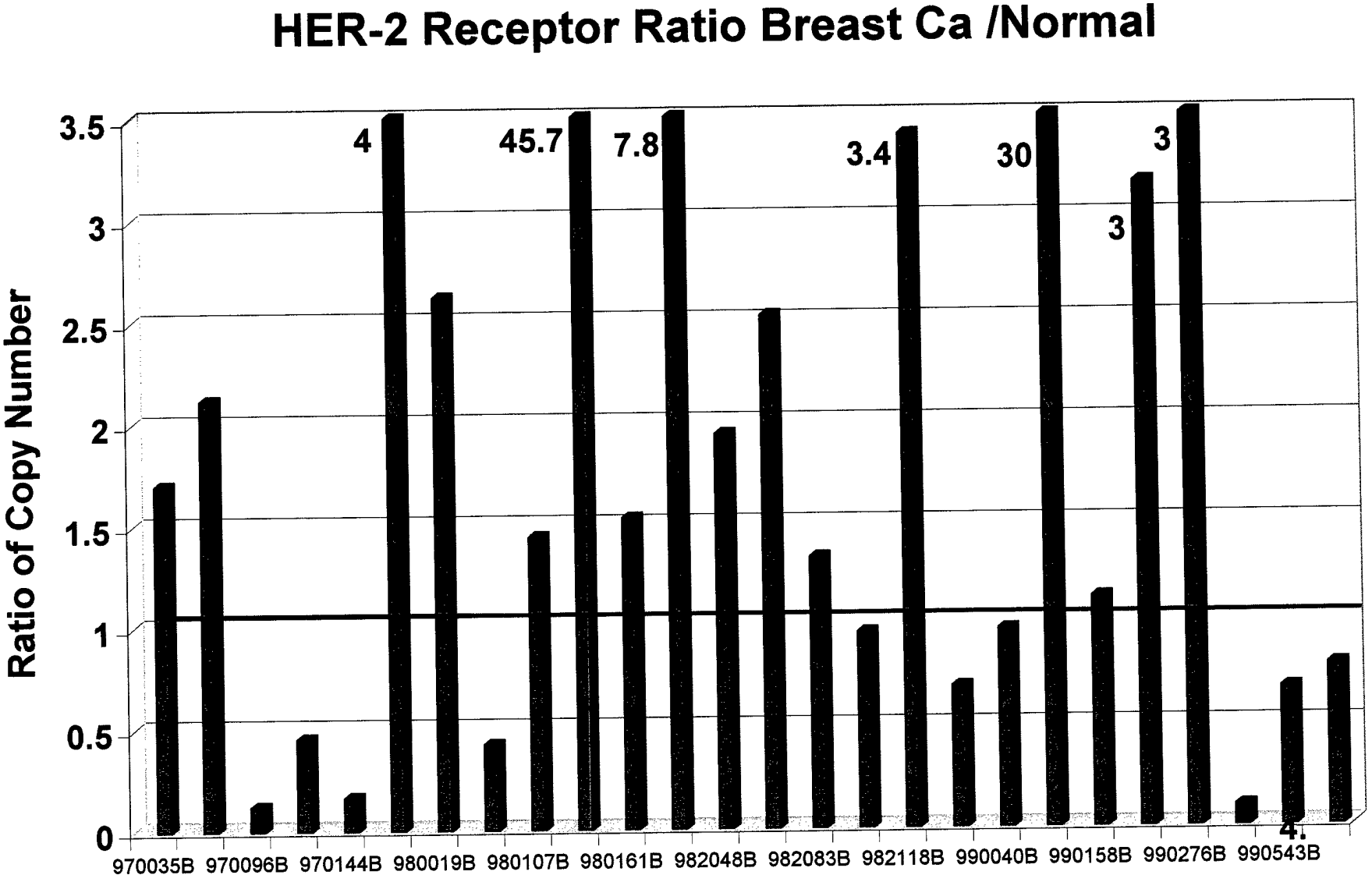
Ribogreen assay: Standard curve



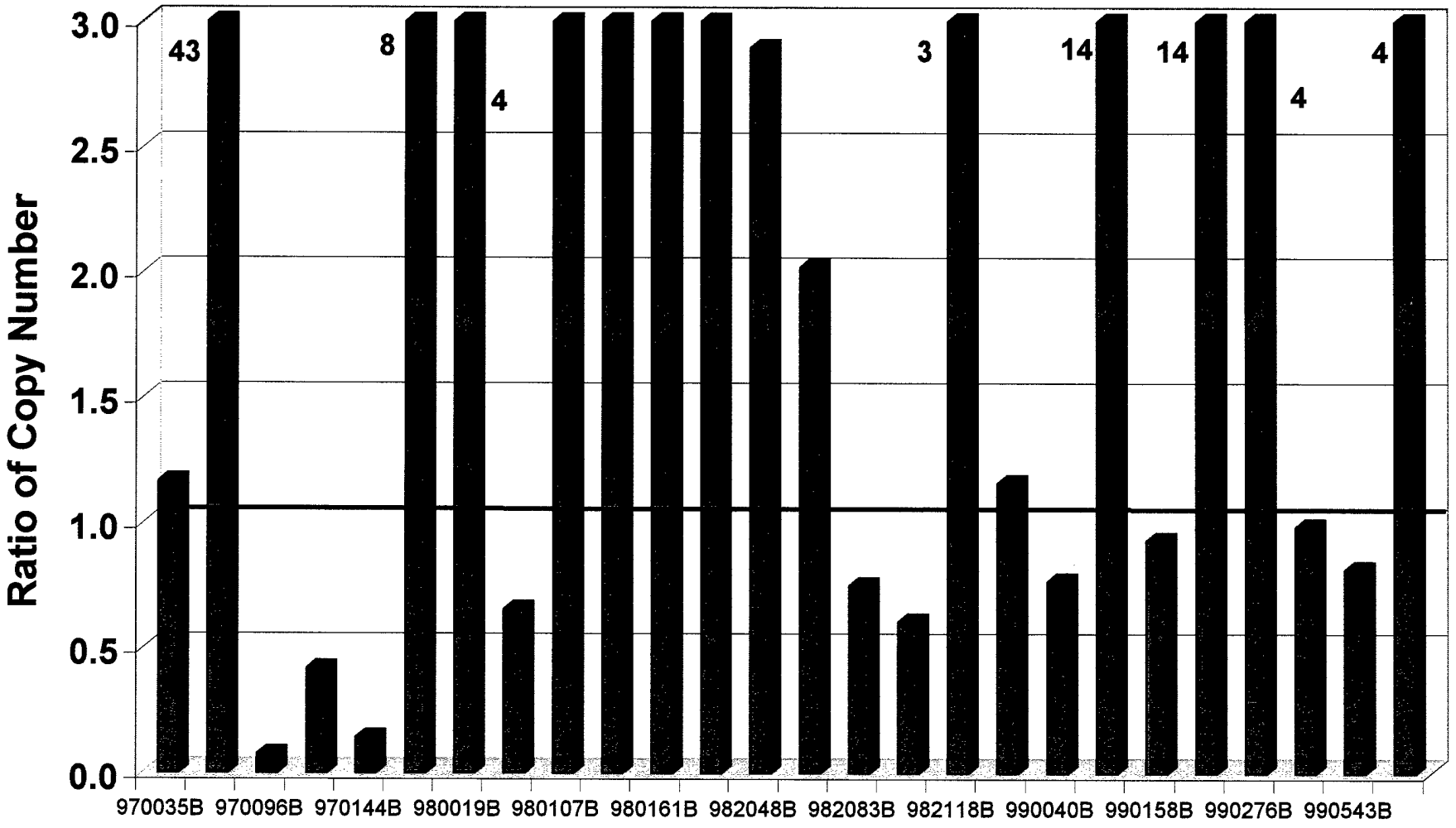
Real Time PCR: Amplification plot

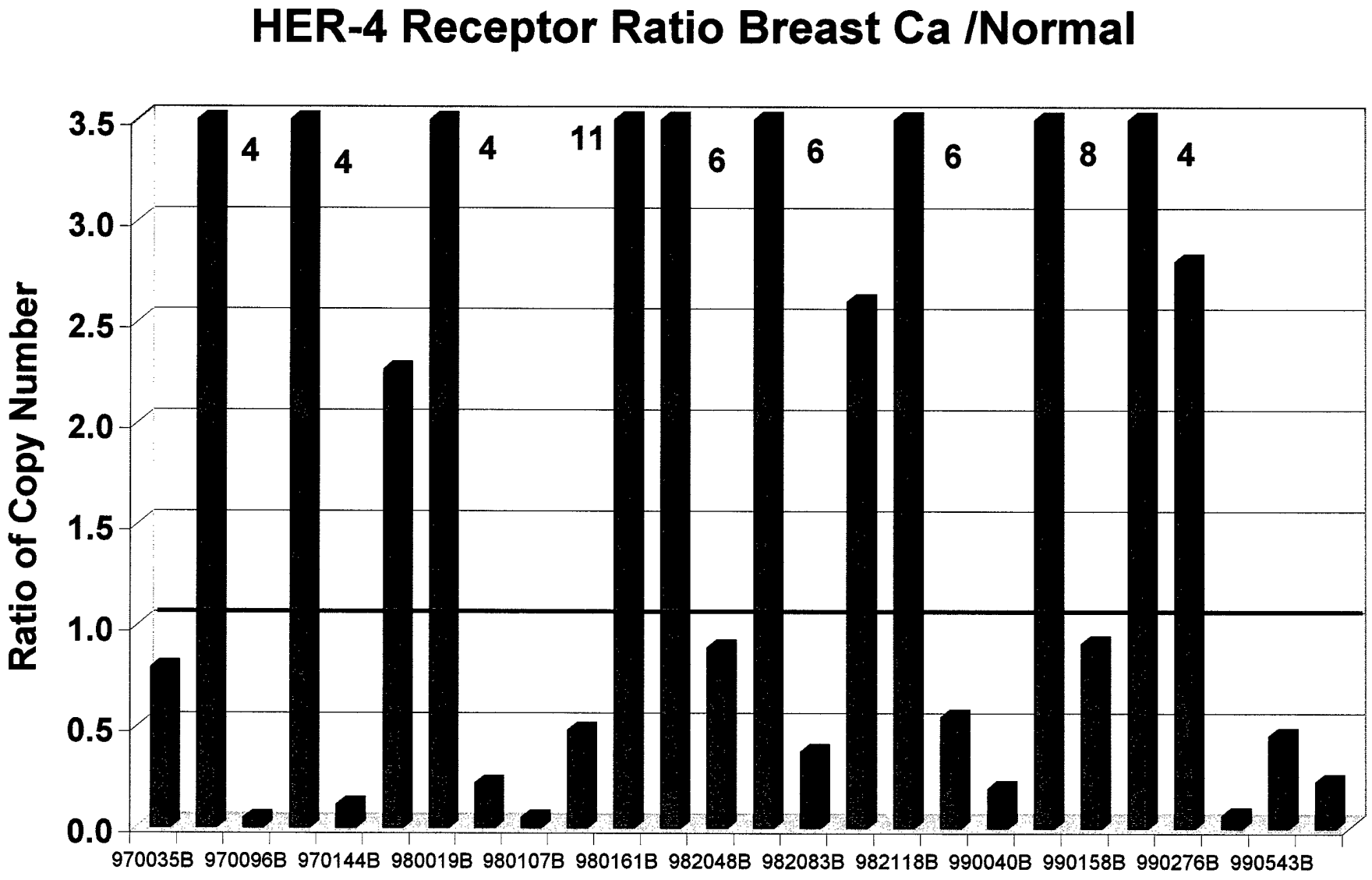



HER-1 Receptor Ratio Breast Ca /Normal
Ratio of Copy Number
3.5
3
2.5
2
1.5
1
0.5
0
3
27
970035B
970096B
970144B
980019B
980107B
980161B
982048B
982083B
982118B
990040B
990158B
990276B
990543B

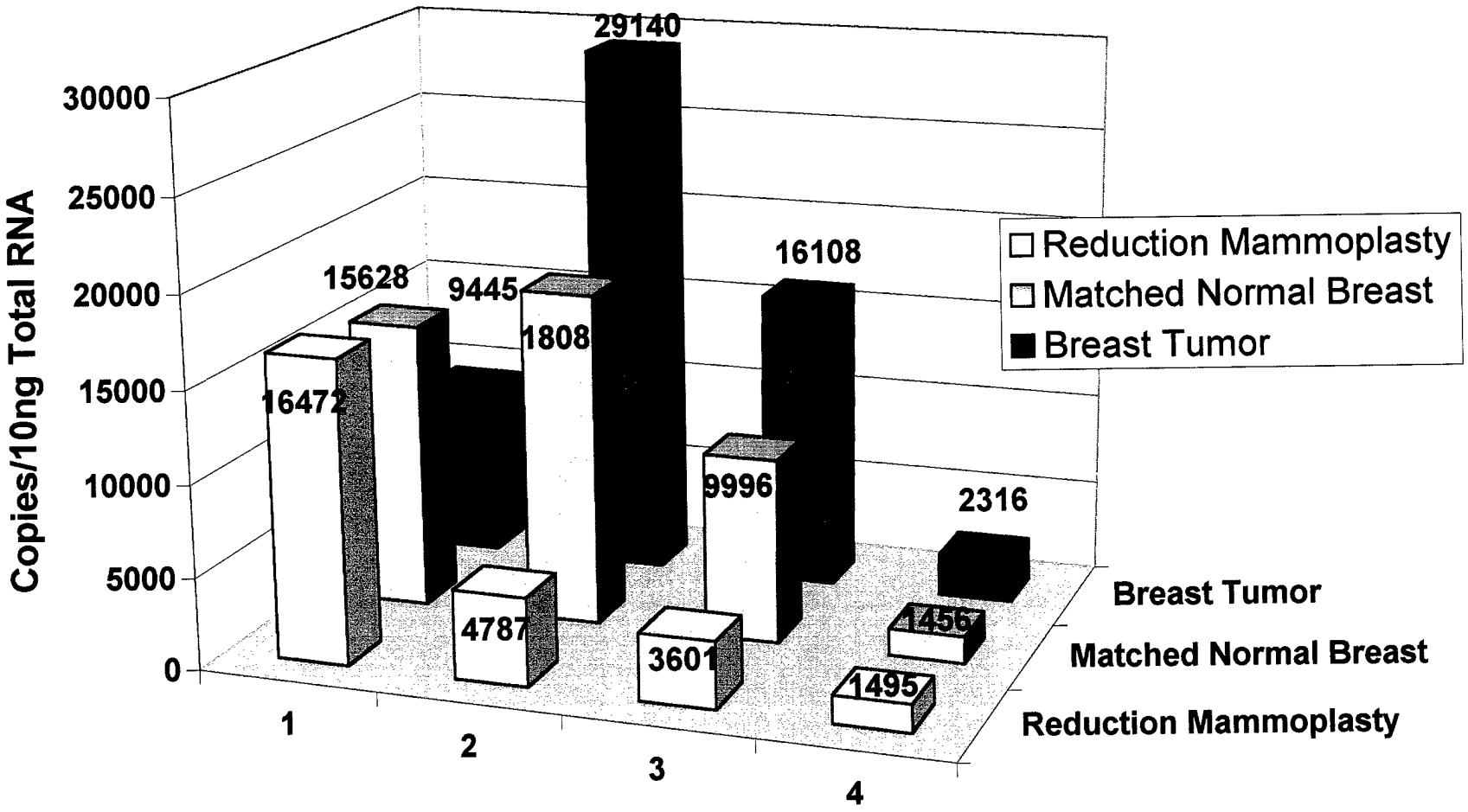

HER-2 Receptor Ratio Breast Ca /Normal
Ratio of Copy Number
3.5
3
2.5
2
1.5
1
0.5
0
4
45.7
7.8
3.4
30
3
3
4.
970035B
970096B
970144B
980019B
980107B
980161B
982048B
982083B
982118B
990040B
990158B
990276B
990543B

HER-3 Receptor Ratio Breast Ca /Normal

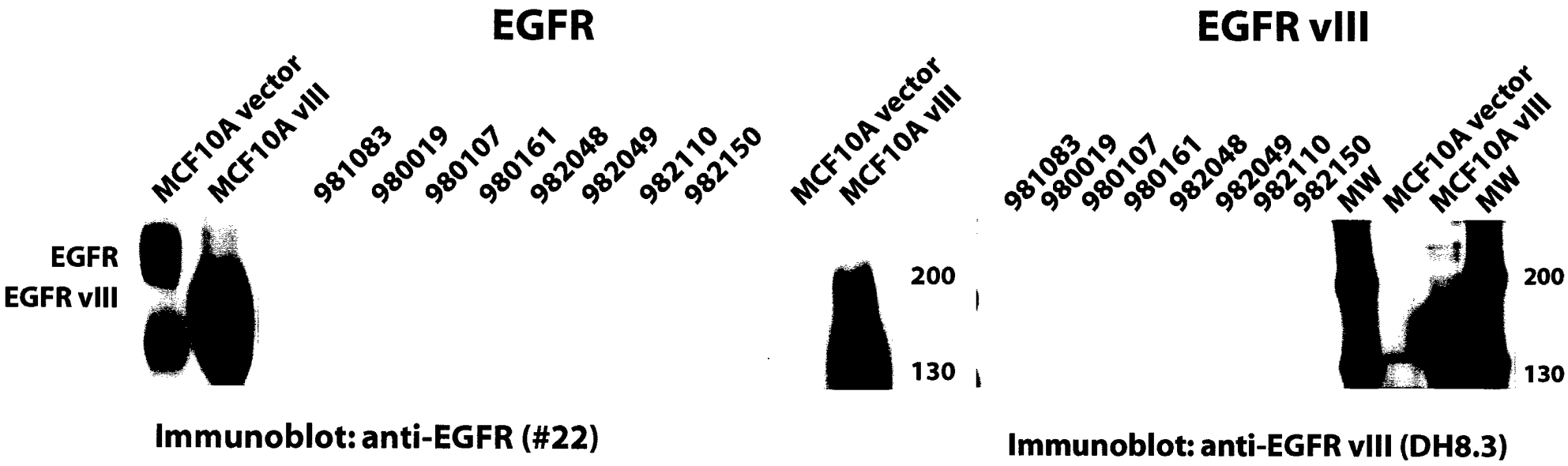



HER-4 Receptor Ratio Breast Ca /Normal
Ratio of Copy Number
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4
4
4
11
6
6
6
8
4
970035B
970096B
970144B
980019B
980107B
980161B
982048B
982083B
982118B
990040B
990158B
990276B
990543B

Average Number of Copies Her 1-4 in Breast Tissue

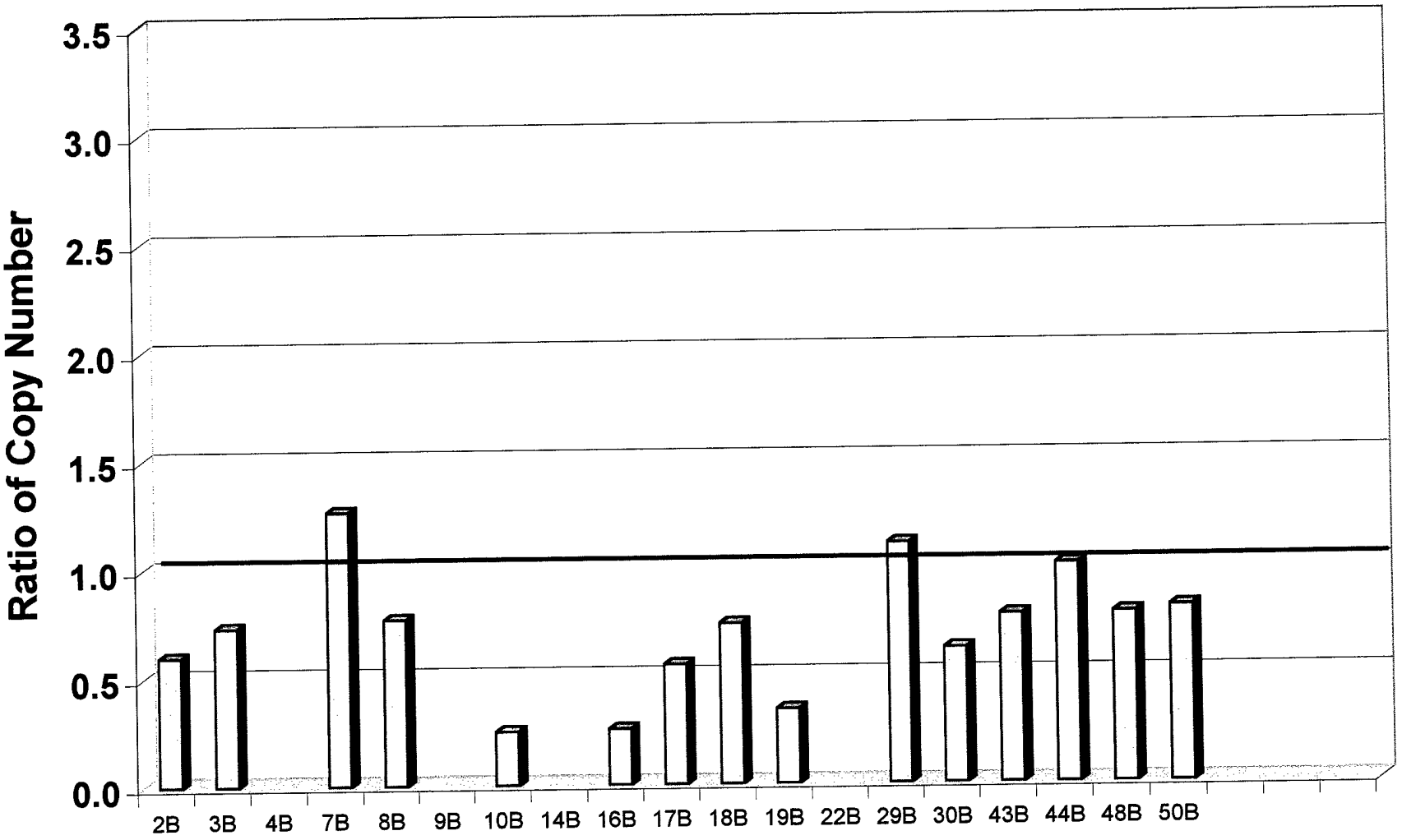


Immunoanalysis of Breast Tumors

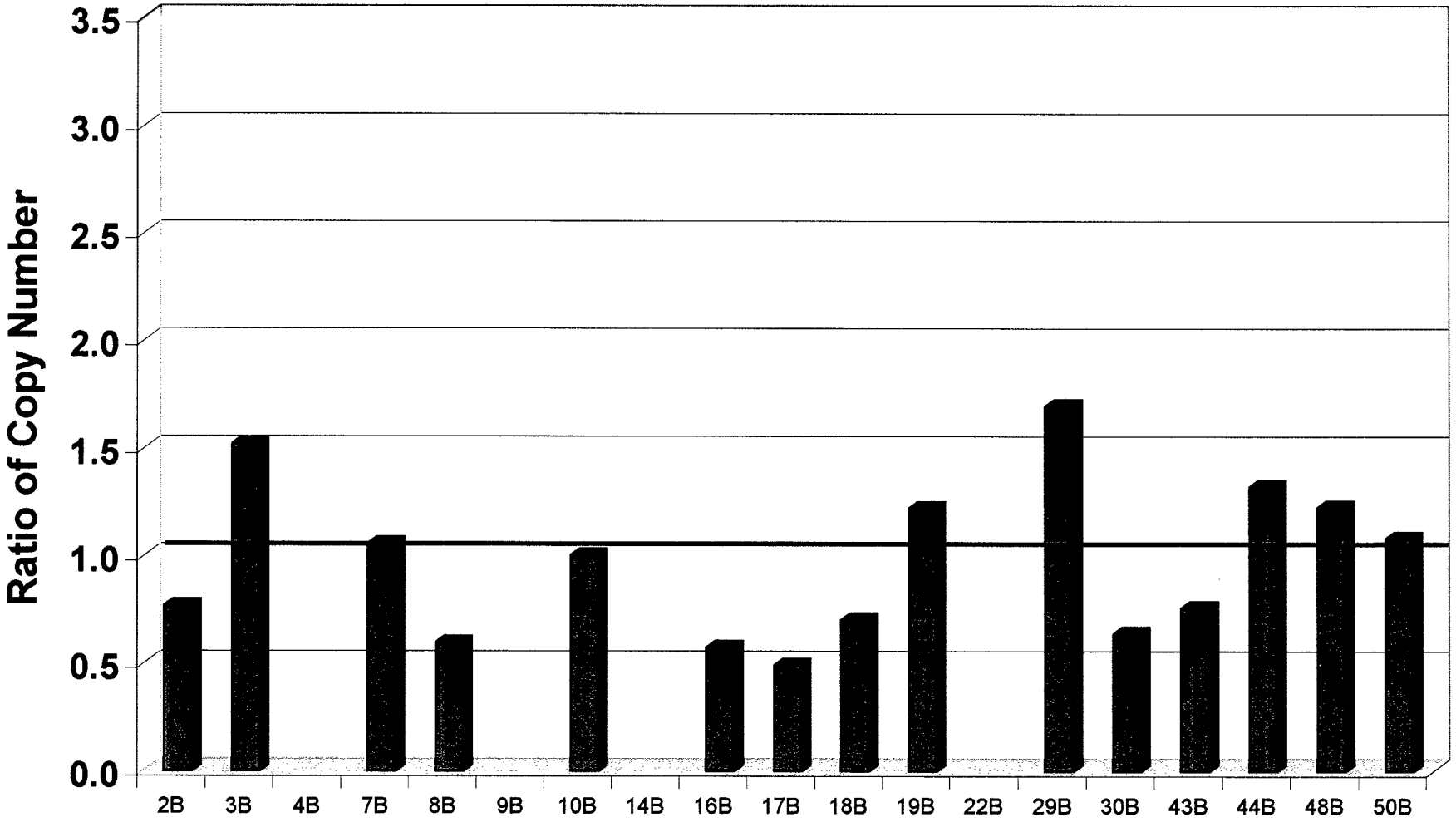


500 μg of protein immunoprecipitated with anti-EGFR (#22)

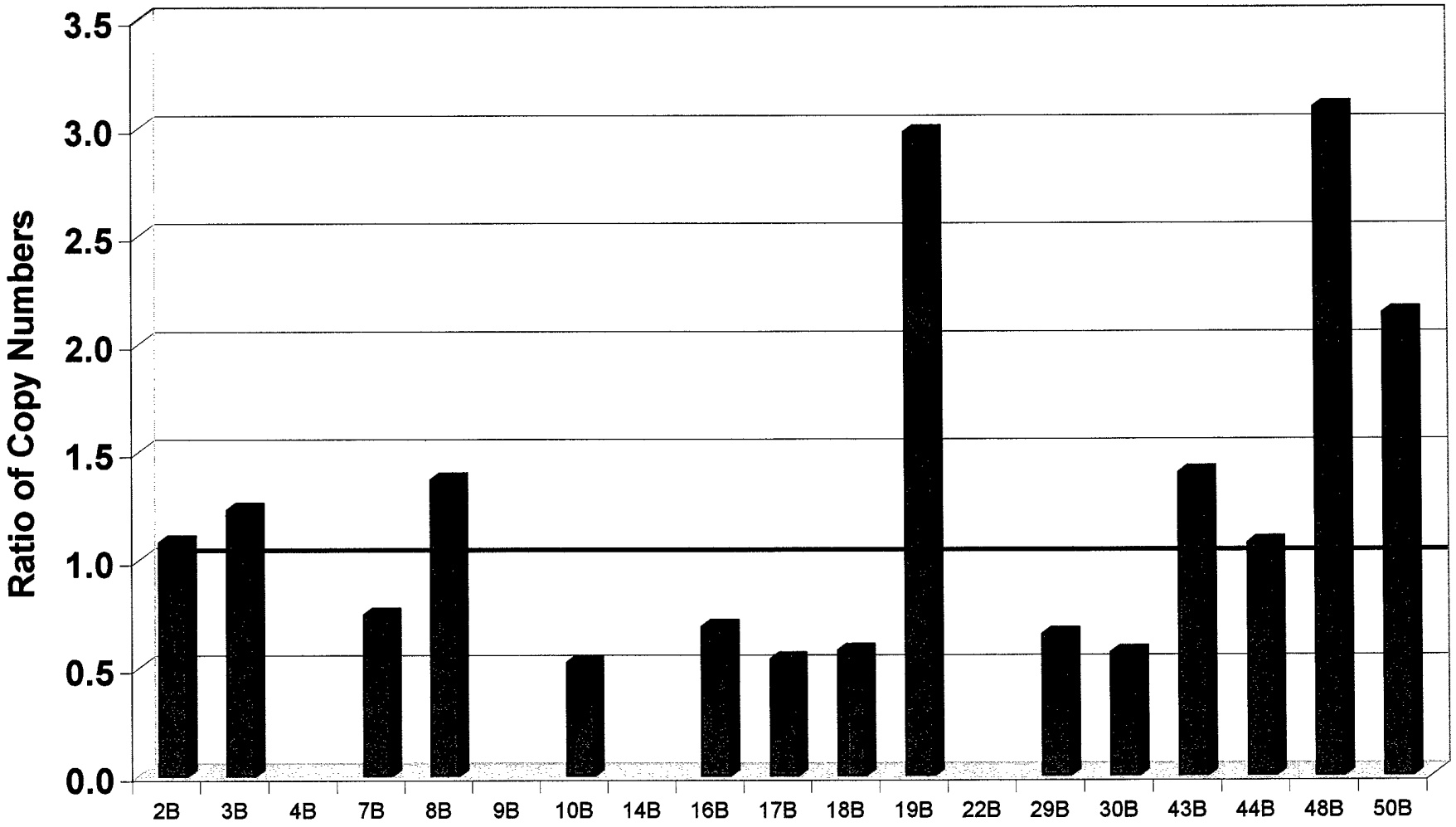
HER-1 Receptor Ratio AD Prostate Ca /Normal



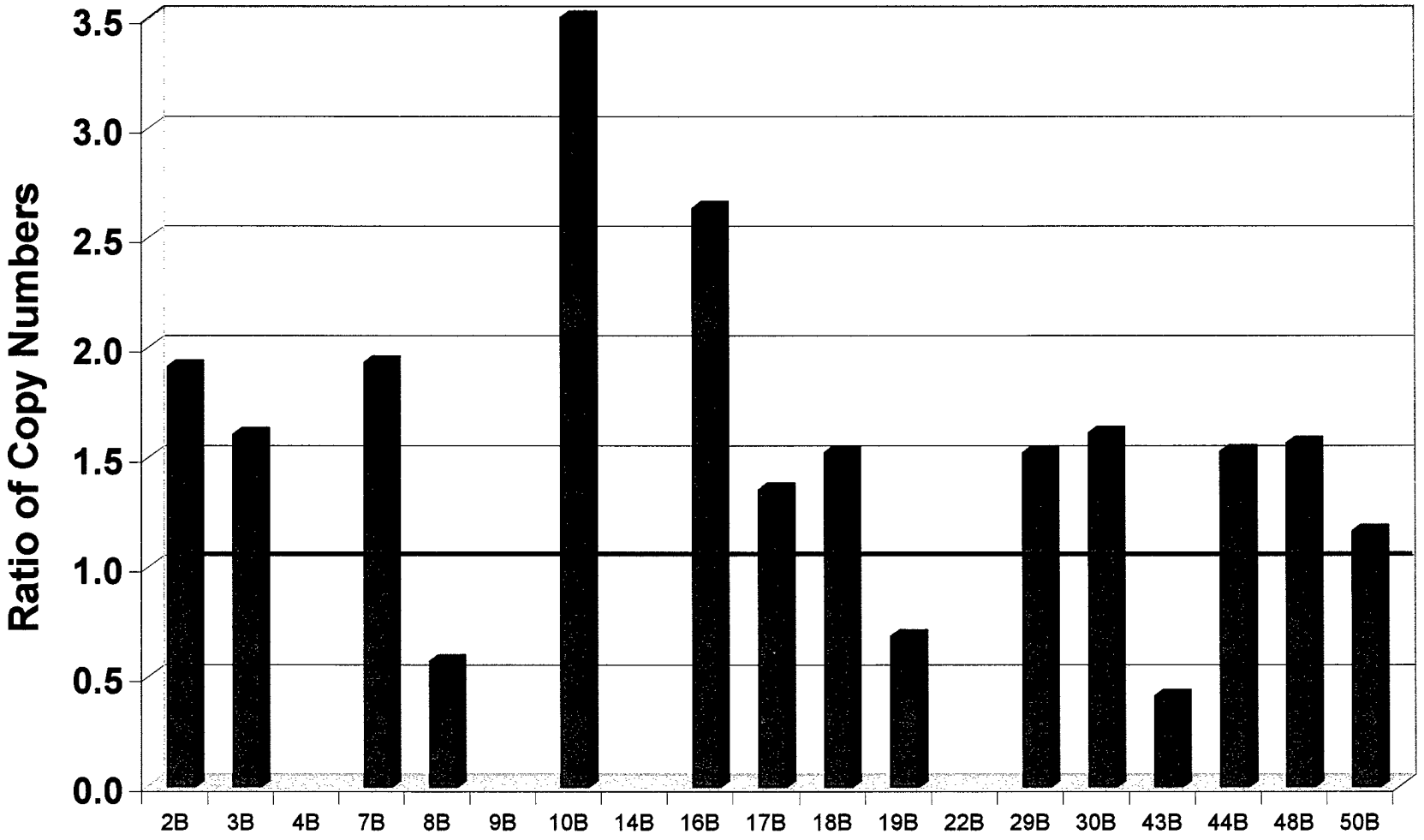
HER-2 Receptor Ratio AD-Prostate Ca / Normal

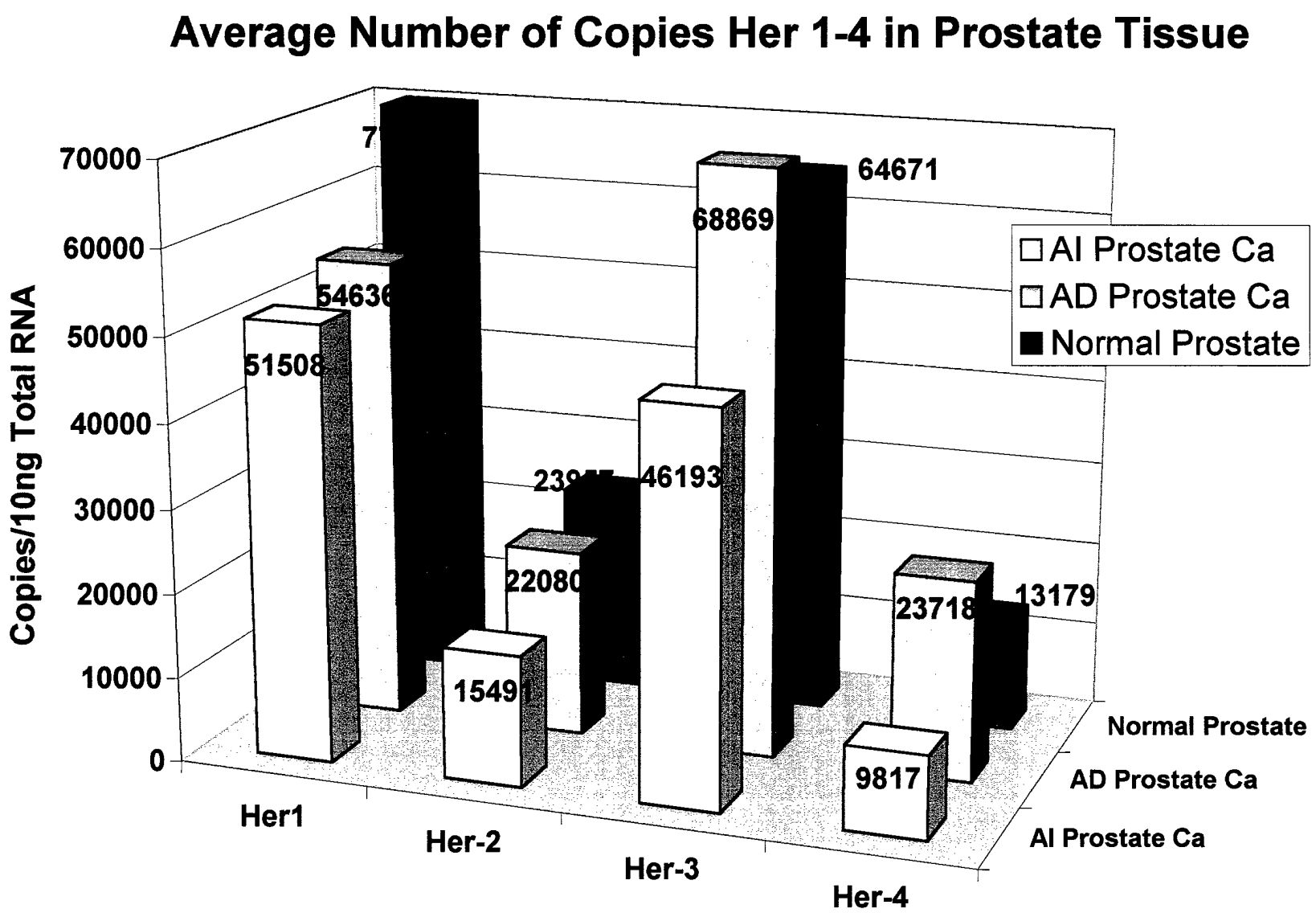


HER-3 Receptor Ratio AD-Prostate Ca / Normal

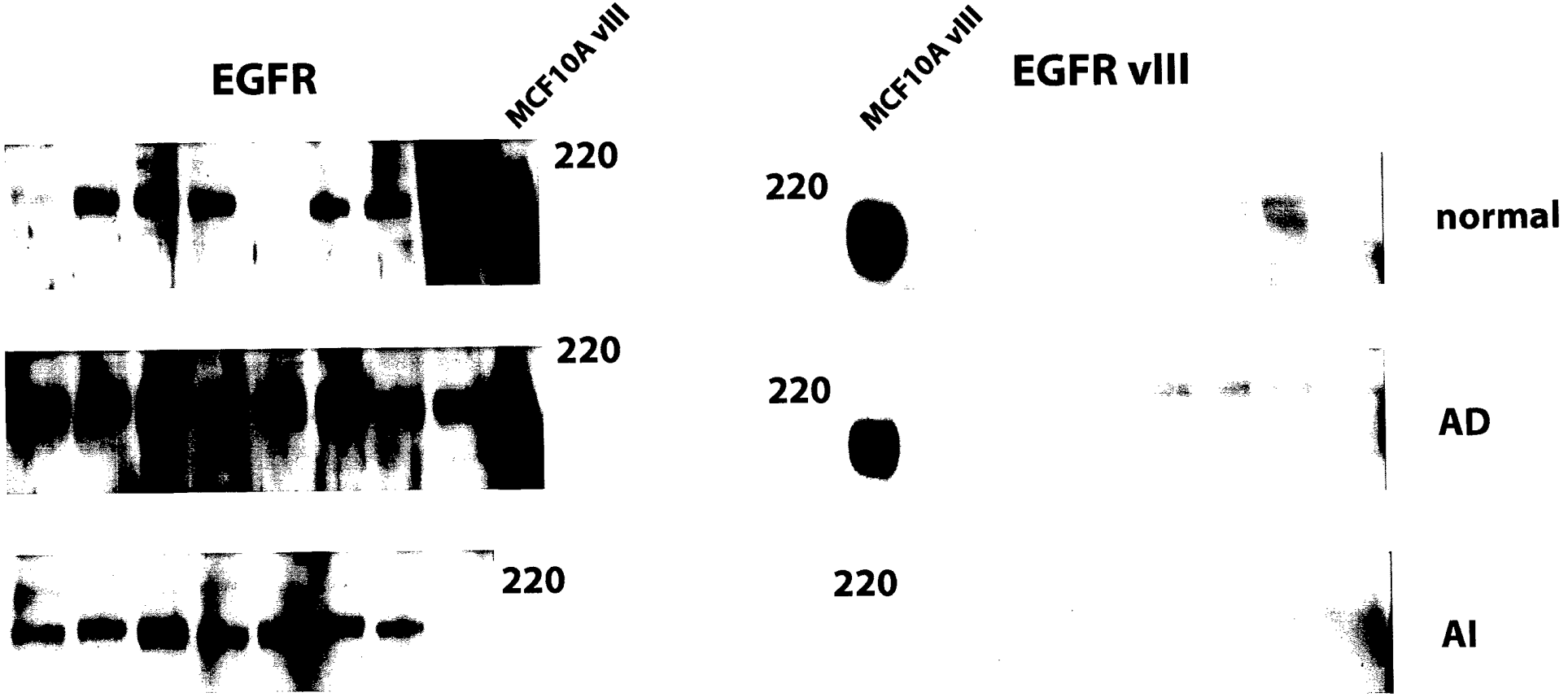


HER-4 Receptor Ratio AD-Prostate Ca / Normal




Average Number of Copies Her 1-4 in Prostate Tissue
Copies/10ng Total RNA
70000
60000
50000
40000
30000
20000
10000
0
AI Prostate Ca
AD Prostate Ca
Normal Prostate
51508
64671
68869
46193
22080
23718
13179
9817
Her1
Her-2
Her-3
Her-4
Normal Prostate
AD Prostate Ca
AI Prostate Ca

Immunoanalysis of Prostate Tumors

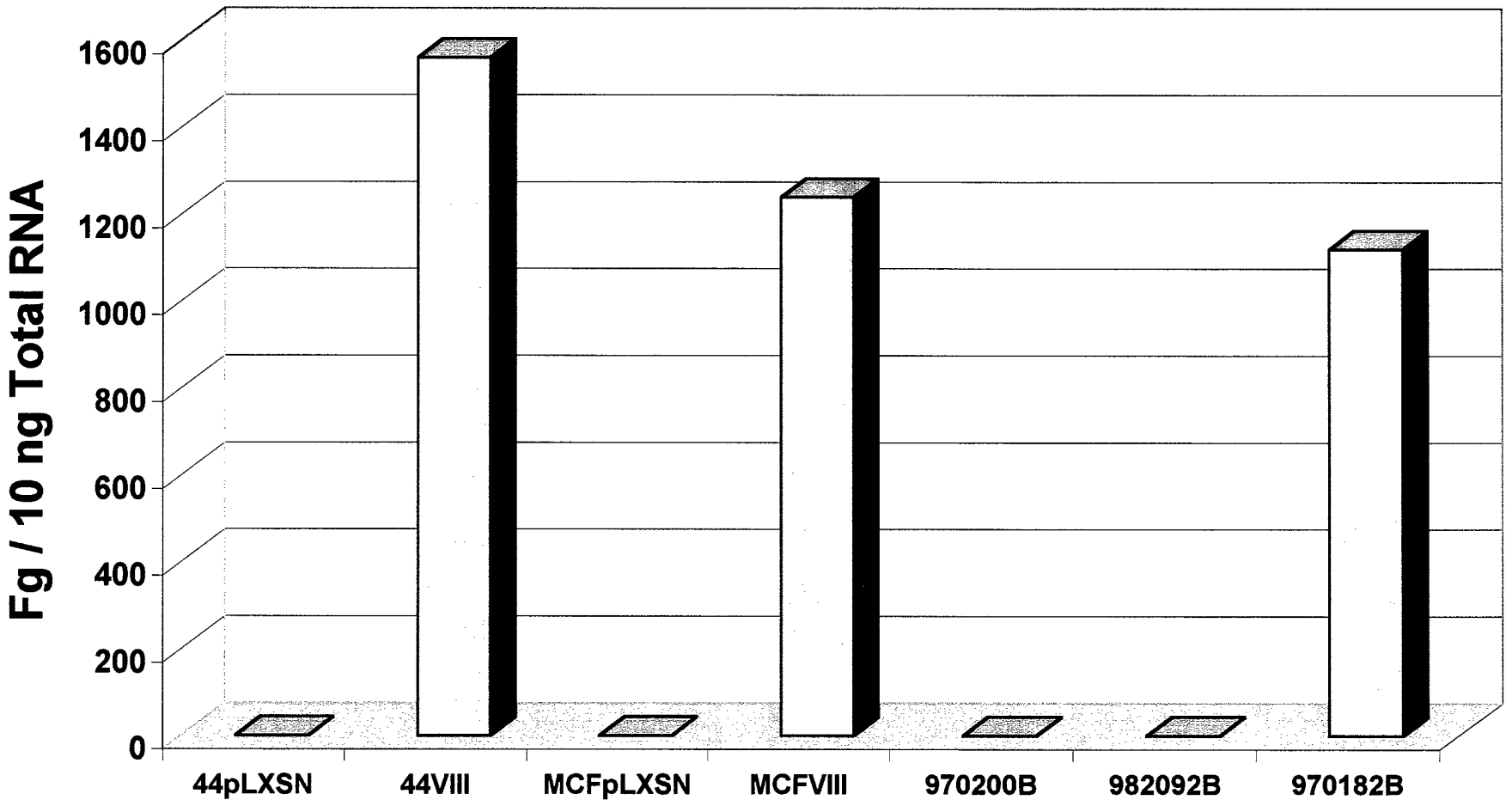


Immunoblot: anti-EGFR (#22)

Immunoblot: anti-EGFR vIII (DH8.3)

50 μg of protein in RIPA buffer

EGFR vIII RNA expression: Cell lines and Gliomas



DISCUSSION

RNA QUALITY: A likely source of inaccurate data when studying gene expression in human tumors is the quality of the RNA in the tissue samples. The Lineberger Comprehensive Cancer Center has administered a medical center tissue procurement facility 10 years. Our extensive quality assurance protocols including sample collection in the Operating Room warrant the confidence we have in the quality of the frozen samples. To further reduce risk of RNA degradation we selected tissue samples banked less than 2 years. The samples were thawed only once and total-RNA was extracted with a guanidinium isothiocyanate based protocol. Purity of extracted RNA was determined by 260/280-absorbance ratios and samples were stored in RNA Storage Solution (Ambion) at –80 °C.

SENSITIVITY OF REAL TIME FLUORESCENT QUANTITATIVE PCR (FQ-PCR): Our laboratory has 3 years of experience with FQ-PCR. The dynamic range curves for Her1-4 and vIII attest to the exquisite sensitivity of this technology that permits us to detect from 1 femtogram to 200,000 femtograms of HER1-4 and vIII messages. We assayed 40ng of total-RNA per well; assuming 3% of total-RNA is mRNA, we expected 1.2×10^6 femtograms of mRNA per well. As any one message represents about 0.01% of the total message pool, we expected 100 fgs. of vIII mRNA per well, a quantity 100 fold higher than our lower limit of detection.

SPECIFICITY OF FQ-PCR: FQ-PCR combines two oligonucleotides flanking a third fluorophore-labeled probe as a mode of detection, resulting in unparalleled detection specificity. EGFR is the message we would most expect to cross-react with oligos designed to bind vIII. Indeed, Olapade [10] reports finding vIII mRNA in prostate carcinomas using three oligonucleotide sets that cross-react with both EGFR and vIII. Of our oligo/probe set only the 3' vIII specific oligonucleotide binds to EGFR while the 5' oligo and the probe are vIII specific. Using this vIII oligo/probe set we were unable to detect 8000 fg of EGFR

sRNA (data not shown), further attesting to its specificity. Despite tremendous sensitivity, FQ-PCR affords a second level of specificity because detection is carried out in a single reaction vessel inclusive off reverse transcription and one set of PCR (40 cycles). In contrast Olapade used 3 sets of sequential nested amplifications, each requiring 40 cycles of PCR.

FQ-PCR VIII DETECTION OF POSITIVE CONTROLS: We *in vitro* transcribed EGFR and vIII sRNA, and used 3 fold dilutions of these specific messages to construct absolute standard curves included in each of the reaction plates. We easily detected vIII expression in SUM44 and MCF10A breast cancer cell lines infected with pLXSN-vIII. To test our ability to detect vIII in human tissues we extracted RNA from three gliomas frozen in our tissue bank. One of the tumors revealed vIII mRNA expression of 1000fg per 40ng total-RNA, similar to the levels detected for the pLXSN-vIII infected cell lines.

EGFR AND EGFR-VIII MRNA EXPRESSION IN BREAST AND PROSTATE CARCINOMAS: EGFR mRNA is not overexpressed in either breast or prostate carcinomas. Indeed the ratio of EGFR mRNA level for prostate carcinomas to matched normal prostate was less than one in 13 of 15 matched patient sets. Similarly, 21 of 26 breast carcinomas had EGFR mRNA ratio less than one. Only two of 26 breast carcinomas expressed EGFR levels three times greater than matched normal breast. We were also unable to detect any vIII expression in breast or prostate carcinoma samples. These results are consistent with the finding that vIII is expressed only in those gliomas that exhibit EGFR gene amplification. Similar data is found for HER-2 where gene amplification is uniformly found in tumors overexpressing HER-2 protein.

IMMUNOANALYSIS: To correlate our mRNA findings with protein expression we used immunoanalysis. We have extensively characterized a polyclonal rabbit antiserum (#22) that binds the carboxyl-terminus of EGFR. This antiserum easily immunoprecipitates both full length EGFR and vIII. We used the commercial anti- vIII monoclonal antibody DH8.3 to detect truncated receptor protein expression in MCF10A-pLXSN-vIII infected cell lines, prostate tumors and breast tumors. Prostate

samples were tested by PAGE of 50ug of protein lysate in RIPA while 500ug of breast carcinoma tumor lysates were immunoprecipatated with anti-EGFR(#22) and PAGE resolved prior to blotting. Both breast and prostate carcinomas revealed expression of full length EGFR. We were also easily able to detect vIII expression in MCF10A-vIII transfected cell line. None of the tumor specimens revealed expression of vIII. In the prostate carcinoma blots the anti-vIII monoclonal antibody appeared to cross-react with a band likely to represent full length EGFR that may explain the reported immunohistochemical findings.

Conclusions

1- The EGFR mRNA is not overexpressed in prostate and infrequently in breast carcinomas.
2- EGFR-vIII mRNA and truncated receptor protein is not expressed in breast or prostate carcinomas.

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